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**Study Of The Central Corticotrophin-Releasing Factor System Using The 2-
Deoxyglucose Method For Measurement Of Local Cerebral Glucose
Utilisation**

Geoffrey Iain Warnock

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

December 2007

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A handwritten signature in black ink, appearing to read 'G I Warnock', with a long horizontal flourish extending to the right.

This thesis has been approved by Johnson & Johnson Pharmaceutical Research
& Development for general release.

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List of Abbreviations

μCi	microcurie
μm	micrometer
2DG	2-deoxyglucose
2DG-6-P	2-deoxyglucose-6-phosphate
5-HT	5-hydroxytryptamine
5-HIAA	5-hydroxyindoleacetic acid
7	facial nucleus
12	hypoglossal nucleus
A1, A5	noradrenaline-containing cell groups
ac	anterior commissure
ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
Amb	ambiguus nucleus
ANOVA	analysis of variance
AON	anterior olfactory nucleus
Apit	anterior pituitary
Arc	arcuate nucleus
aSVG	antisauvagine-30
ATP	adenosine triphosphate
Basal G	basal ganglia
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
bpm	beats per minute
CA1, 2, 3	fields CA1, 2, 3, of the hippocampus
cc	corpus callosum
cDNA	complementary deoxyribonucleic acid
Ce	cerebellum
CeA	central nucleus of the amygdala
CG	central grey matter
CingCx	cingulate cortex
CNS	central nervous system
CoA	cortical nucleus of the amygdala
CRF	corticotropin-releasing factor
CRF1KO	mice lacking a functional CRF ₁ receptor
CRF-BP	CRF-binding protein
CSF	cerebrospinal fluid
DBB	diagonal band of broca
DeepN	deep nuclei
DG	dentate gyrus
DR	dorsal raphe
DVC	dorsal motor nucleus of the vagus
EDTA	ethylenediaminetetraacetic acid
EW	edinger westphal nucleus
FDG	fluorodeoxyglucose
FrCx	frontal cortex
GABA	gamma aminobutyric acid
GLUT	glucose transporter

h	human
Hb	hindbrain
Hipp	hippocampus
HPA	hypothalamic-pituitary-adrenocortical axis
Hy	hypothalamus
IC	inferior colliculi
ICV	intracerebroventricular
IP	intraperitoneal
IV	intravenous
K _i	inhibitory binding constant
LC	locus coeruleus
LCGU	local cerebral glucose utilisation
LDTg	laterodorsal tegmental nucleus
LH	lateral hypothalamus
LS	lateral septum
LSO	lateral superior olive
m	mouse
MeA	medial nucleus of the amygdala
MePO	median preoptic nucleus
mfb	median forebrain bundle
mg/dl	milligrams per decilitre
mg/kg	milligrams per kilogram
mg/kg/day	milligrams per kilogram per day
min	minute
mmHg	millimetres mercury
mmol	millimole
mPFC	medial prefrontal cortex
MPO	median preoptic area
MR	median raphe
mRNA	messenger ribonucleic acid
MS	medial septum
MVN	medial vestibular nucleus
nCi/mg	nanocuries per milligram
nCi/ml	nanocuries per millilitre
ng/ml	nanograms per millilitre
nM	nanomolar
NTS	nucleus of the solitary tract
o	ovine
OB	olfactory bulb
OccCx	occipital cortex
PAG	periaqueductal grey
ParCx	parietal cortex
PB	parabrachial nucleus
PE	polyethylene
PET	positron emission tomography
PFA	perifornical area
POMC	proopiomelanocortin
POR	perioculomotor nucleus
Ppit	posterior pituitary
PPTg	pendunculo pontine tegmental nucleus

Pta	parietal association cortex
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus of the hypothalamus
r	rat
R	red nucleus
rCBF	regional cerebral blood flow
RIA	radio immuno assay
RN	raphe nuclei
SC	superior colliculi / subcutaneous
SEM	standard error of the mean
Sept	septum
SI	substantia inominata
SN	substantia nigra
SON	supraoptic nucleus
Sp5n	spinal trigeminus nucleus
SPO	superior paraolivary nucleus
SSRI	selective serotonin reuptake inhibitor
Temp	temporal dissected region
Th/Thal	thalamus
Ucn	Urocortin
SCP	Stresscopin
VMH	ventromedial hypothalamus.

Candidate's Declaration

I certify that this thesis is my own work, except where noted, and has not been submitted for any degree other than that of Doctor of Philosophy at the University of Bath.

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Acknowledgments

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Abstract

Stress is defined as a challenge to homeostatic equilibrium by physical or psychological events, generating a coping response consisting of central and peripheral changes, with the aim of exerting control over the threatening events. Corticotropin-releasing factor (CRF) is well known as a hypothalamic factor which controls the hypothalamic-pituitary-adrenocortical (HPA) axis during basal activity and stress. CRF also serves a neurotransmitter function in the brain, where it is implicated in a range of stress-related behaviours.

The measurement of local cerebral glucose utilisation (LCGU) using radiolabelled 2-deoxy-D-glucose (2DG) provides an estimate of cellular activity in the brain. 2DG competes with glucose in its metabolic pathway, but is not fully metabolised, instead accumulating within astrocytes where it can be quantified.

After consideration of available modifications to the LCGU technique, the effect of manipulating the CRF system on LCGU was studied, in order to test the hypothesis that CRF and other endogenously expressed CRF-related peptides would induce different patterns of LCGU, and to examine the involvement of CRF receptors in any response. The CRF₁ receptor has been implicated in the mediation of stress- and anxiety-related behaviour, while recent evidence has suggested a role for CRF₂ in mediating the delayed effects of stress, although it has previously been postulated that CRF₂ may be involved in the attenuation of stress-related behaviour.

CRF and the endogenous CRF-related peptide Urocortin 1 both induced increases in LCGU in a number of brain regions associated with the CRF system, with concomitant activation of the HPA axis. CRF induced increases in LCGU in the dissected hypothalamus, thalamus, cerebellum and hippocampus, while Urocortin 1 induced a significant increase in LCGU in a dissected hindbrain region, with trend-like effects in frontal cortex and hippocampus. These regions contain components of the CRF system, or receive projections from regions involved in the CRF system, and have been implicated in stress-related function. The effects of CRF on LCGU appear to be mediated by the CRF₂ receptor, as

they were abolished by the selective CRF₂ antagonist antisauvagine-30, but persisted in mice lacking CRF₁ and were unaffected by a selective CRF₁ antagonist. However, neither of the endogenous CRF-related peptides selective for CRF₂, Urocortin 2 and Stresscopin, affected LCGU, which may indicate ligand-specific effects within the CRF system.

In contrast to the effects of CRF, restraint stress reduced LCGU, while activating the HPA axis, and this response was unaffected by a selective CRF₁ antagonist. This data suggests that the role of CRF receptors in restraint-induced LCGU changes may be overshadowed by effects on other neurotransmitter systems.

These studies support the hypothesis that CRF and other endogenously expressed CRF-related peptides would induce different patterns of LCGU, and highlight the involvement of particular brain regions in the response to CRF receptor stimulation. Furthermore, these studies provide evidence suggesting ligand-specific effects within the CRF system.

Chapter 1 – General Introduction

Corticotropin-Releasing Factor: An Overview

Corticotropin-releasing factor (CRF), a 41-residue straight-chain peptide isolated initially in 1981 from ovine hypothalamus by Vale and colleagues at the Salk Institute (Vale et al., 1981), is most well known for its role in the hypothalamic-pituitary-adrenocortical (HPA) axis, one of the main effector systems that are activated in numerous species upon exposure to an acute stressor.

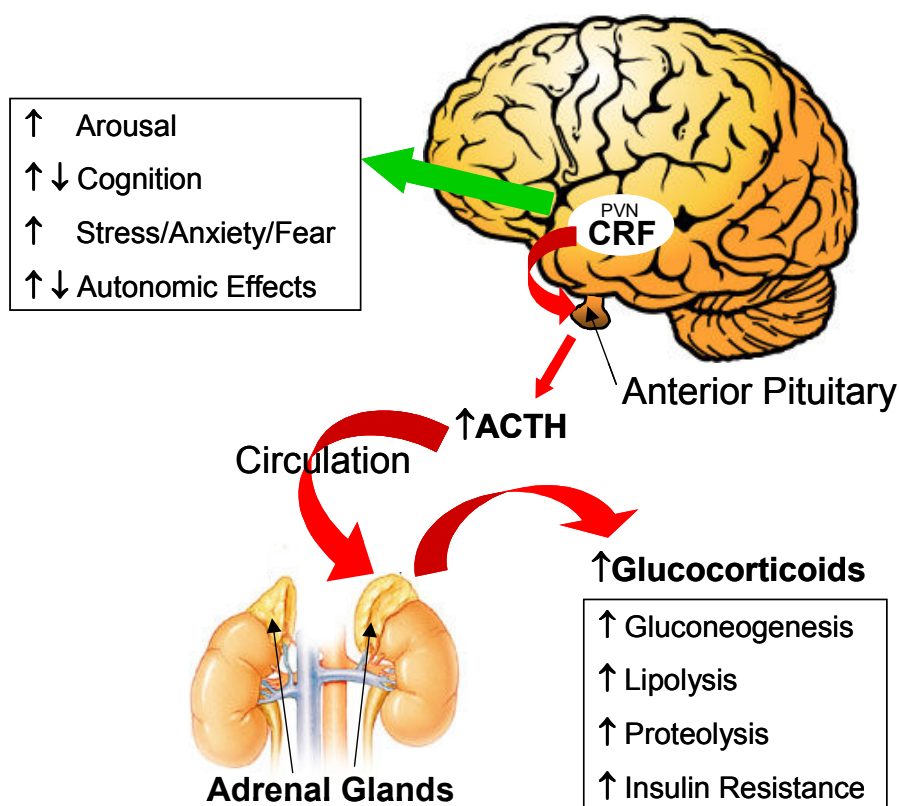
Stress is defined as a challenge to homeostatic equilibrium by physical or psychological events (McEwen, 2003), generating a coping response consisting of central and peripheral changes, with the aim of exerting control over the threatening events. Effective coping implies that a stress response is effectively triggered when required and is terminated appropriately (de Kloet et al., 2005). However, if the stress response is inadequate or prolonged excessively, a series of pathophysiological changes may occur in the brain, immune system and viscera (Musselman and Nemeroff, 2000; Sapolsky, 2000; Mayer and Fanselow, 2003; Sorrells and Sapolsky, 2007), that may ultimately contribute to the development of disorders including depression and post-traumatic stress disorder.

CRF is also expressed throughout the brain, and dysfunction of the CRF system has been implicated in a number of stress-related disorders. Since its discovery, CRF has been linked to numerous endogenous functions, both centrally and peripherally mediated. CRF is implicated in a range of functions that may be affected by stress, including cognitive processes, regulation of food intake and satiety, gastrointestinal motility, vascular tone and development, hearing and cardiac function, demonstrating the vast importance of the CRF family (Bale and Vale, 2004). The CRF system and its hypothesised role in the brain will be discussed in more detail below.

In response to acute physical or psychological stress, parvocellular neurons of the paraventricular hypothalamic nucleus (PVN) produce increased amounts of CRF. CRF enters the portal circulation to regulate proopiomelanocortin (POMC)-derived peptide (mainly adrenocorticotrophic

hormone (ACTH) and beta-endorphin) synthesis and secretion from the anterior pituitary (Rivier and Plotsky, 1986). In turn ACTH enters the circulation and stimulates glucocorticoid (e.g. corticosterone, the glucocorticoid most associated with the stress response in rodents; cortisol in humans) release from the adrenal gland. This extended pathway constitutes the HPA axis (illustrated in Figure 1). Glucocorticoids readily penetrate the blood-brain barrier and exert feedback on the HPA axis.

Figure 1: The HPA axis, with examples of both effects mediated by increased circulating glucocorticoids, and central CRF (Adapted from Holsboer, 1999).



A stress-related CRF pathway has been suggested (Lee and Davis, 1997). It includes CRF input into the central nucleus of the amygdala (CeA), originating in the lateral hypothalamus, dorsal raphe and intrinsic cells of the CeA (Gray, 1993). CRF neurons in the CeA project to the bed nucleus of the stria terminalis (BNST) (Sakanaka et al., 1986), from where there are CRF projections to the PVN, leading to activation of the HPA axis. HPA axis activation results in corticosterone release from the adrenal gland which self-regulates through three major feedback mechanisms: 1) negative feedback at the level of PVN neurons

and anterior pituitary (Swanson and Simmons, 1989); 2) positive feedback at the CeA and the dorsal PVN, from where spinal projections of CRF neurons emerge (Swanson and Simmons, 1989; Gray and Bingaman, 1996) and 3) negative feedback through the ventral hippocampus, which projects to the BNST via the fimbria/fornix (Herman et al., 1992; Cullinan et al., 1993). Negative feedback on the HPA axis may also exist at the level of the medial prefrontal cortex (mPFC), as corticosterone administered directly into the mPFC has been shown to attenuate stress-induced HPA activation (Diorio et al., 1993).

However, CRF, its receptors and related peptides, are not restricted to the pathways of the HPA axis, and its function via these other regions has been linked to a number of behaviours and pathological states. The extended distribution of the CRF system and its postulated roles will be discussed below.

The Distribution of CRF

The hypothalamic PVN is the major site of CRF-containing cell bodies (Merchenthaler, 1984; Swanson and Simmons, 1989) (Figure 2b). These cell bodies send axon terminals to the capillaries of the median eminence, from where CRF enters the portal circulation to regulate pituitary ACTH release. Other CRF neurons of PVN origin project to the brainstem and spinal cord, both of which contain CRF cell bodies and influence behavioural activity and autonomic function (Vale et al., 1981; De Souza, 1995).

In addition to CRF in the PVN, in the rat there are large and discrete populations of CRF neurons in the CeA, the BNST, the hippocampus, the accumbens, the posteromedial thalamic nuclei such as the mediodorsal nuclei, the substantia nigra, the locus coeruleus (LC), the dorsal and the medial raphe nuclei, the periaqueductal grey, the olfactory bulbs, the parabrachial nuclei, the nucleus of the solitary tract (NTS) and the cerebellum (Swanson et al., 1983; Merchenthaler et al., 1984; Merchenthaler, 1984; Sakanaka et al., 1987; Van Bockstaele et al., 1996; Morin et al., 1999). Particularly high densities of CRF-containing neurons are found in the prefrontal and cingulate cortices, and throughout the neocortex, emphasising a possible role of CRF in cognitive processes (Swanson et al., 1983). In humans, CRF-immunoreactivity has been

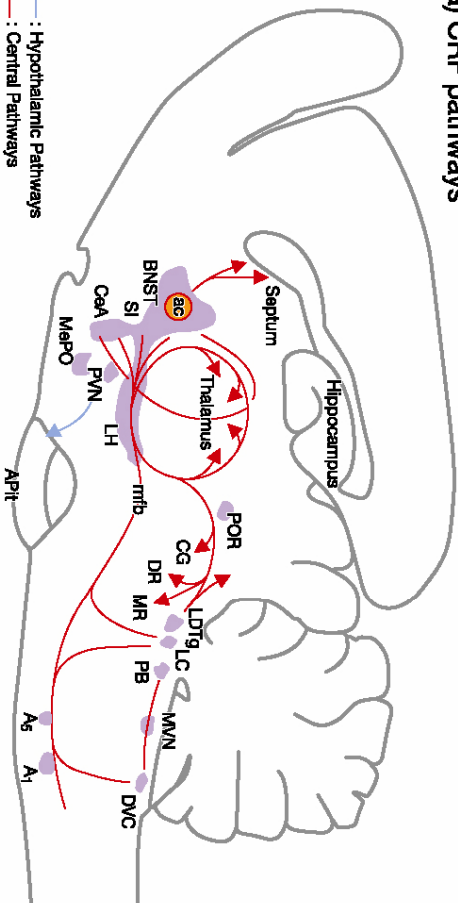
found in the frontal cortex, temporal cortex, occipital cortex, thalamus, hypothalamus, medulla oblongata, and in the cerebellum and low CRF concentrations were also found in the pons (Takahashi et al., 1998).

CRF neurons located in the PVN project to the LC (Valentino et al., 1992) (Figure 2a). Another CRF projection to the LC originates in the BNST (Van Bockstaele et al., 1998), which also sends projections to noradrenergic neurons in the NTS (Gray and Magnuson, 1987). CRF cell bodies and fibres are also associated with the serotonergic neurons at the level of the raphe nuclei (Cummings et al., 1983; Sakanaka et al., 1986; Austin et al., 1997; Ruggiero et al., 1999). However, most extrahypothalamic CRF neurons are located within the CeA, from where CRF neurons project to areas including the PVN (Gray, 1993), BNST (Sakanaka et al., 1986) and the LC (Valentino et al., 1993; Koegler-Muly et al., 1993). Finally, another CRF projection originates in the medial amygdala and innervates the ventromedial hypothalamic nucleus (VMH) (Sakanaka et al., 1986; Gray, 1993).

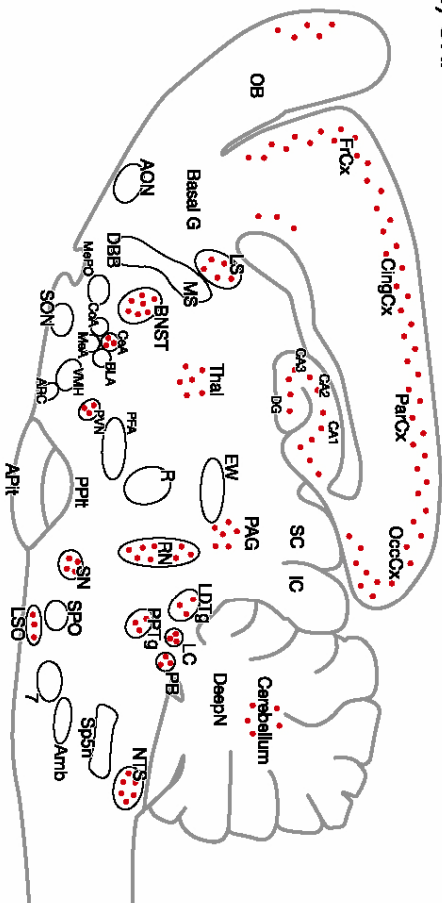
Figure 2: (a) CRF pathways, (b) CRF peptide, (c) CRF receptor mRNA, and (d) Urocortin 1, 2, 3 mRNA distributions in the rodent brain; adapted from (Holmes et al., 2003);

Abbreviations: 7, facial nucleus, 12, hypoglossal nucleus, A1, A5, noradrenaline-containing cell groups, ac, anterior commissure, Amb, ambiguous nucleus, AON, anterior olfactory nucleus, APit, anterior pituitary, Arc, arcuate nucleus, Basal G, basal ganglia, BLA, basolateral amygdala, BNST, bed nucleus of the stria terminalis, CA1, 2, 3, fields CA1, 2, 3, of the hippocampus, cc, corpus callosum, CeA, central nucleus of the amygdala, CG, central grey matter, CingCx, cingulate cortex, CoA, cortical nucleus of the amygdala, DBB, diagonal band of broca, DeepN, deep nuclei, DG, dentate gyrus, DR, dorsal raphe, DVC, dorsal motor nucleus of the vagus, EW, edinger westphal nucleus, FrCx, frontal cortex, Hipp, hippocampus, IC, inferior colliculi, LC, locus coeruleus, LDTg, laterodorsal tegmental nucleus, LH, lateral hypothalamus, LS, lateral septum, LSO, lateral superior olive, MeA, medial nucleus of the amygdala, MePO, median preoptic nucleus, mfb, median forebrain bundle, MPO, median preoptic area, MR, median raphe, MS, medial septum, MVN, medial vestibular nucleus, NTS, nucleus of the solitary tract, OB, olfactory bulb, OccCx, occipital cortex, PAG, periaqueductal grey, ParCx, parietal cortex, PB, parabrachial nucleus, PFA, perifornical area, POR, periculusomotor nucleus, PPit, posterior pituitary, PPTg, pedunculo-pontine tegmental nucleus, PVN, paraventricular nucleus of the hypothalamus, R, red nucleus, RN, raphe nuclei, SC, superior colliculi, SI, substantia innominata, SN, substantia nigra, SON, supraoptic nucleus, Sp5n, spinal trigeminal nucleus, SPO, superior paraolivary nucleus, Thal, thalamus, VMH, ventromedial hypothalamus.

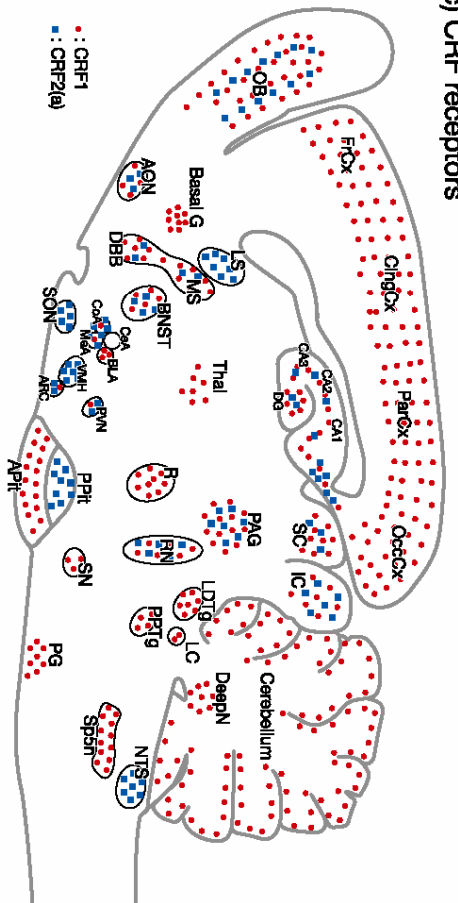
(a) CRF pathways



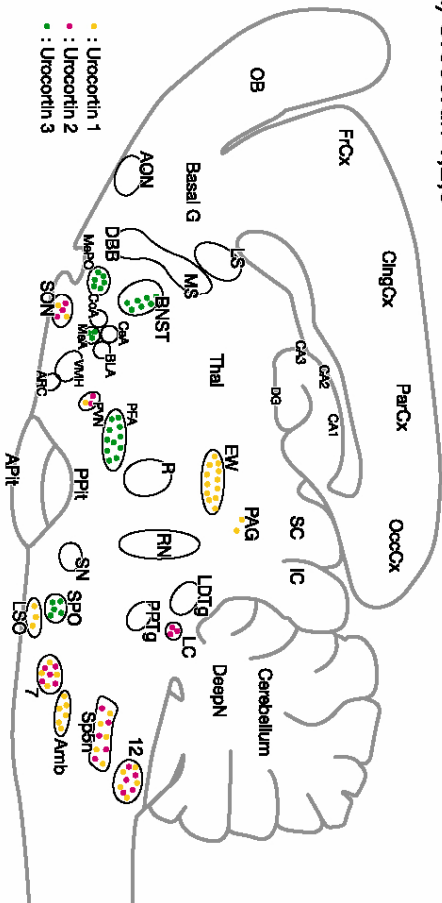
(b) CRF



(c) CRF receptors



(d) Urocortin 1,2,3



CRF receptors

Two primary subtypes of CRF receptor have been discovered, CRF₁, the most abundant in the brain, and CRF₂, of which two major splice-variants have been reported, CRF_{2(a)}, the primary variant in the brain and CRF_{2(b)}, predominantly expressed in the periphery. CRF is a non-specific agonist at both receptors, although it has a greater affinity for CRF₁ (Table 2).

The CRF₁ receptor has been cloned from a variety of species, including human (Chen et al., 1993; Vita et al., 1993), mouse (Vita et al., 1993), rat (Perrin et al., 1993; Chang et al., 1993), chicken (Yu et al., 1996), frog (Dautzenberg et al., 1997), sheep (Myers et al., 1998), tree shrew (Palchaudhuri et al., 1998) and fish (Arai et al., 2001; Pohl et al., 2001).

A number of splice variants of the CRF₁ receptor cDNA have been identified (Chen et al., 1993; Chang et al., 1993; Ross et al., 1994; Myers et al., 1998; Grammatopoulos et al., 1999), but these have not been shown to encode functional receptors *in vivo* due to their low binding affinity or lack of activation in recombinant systems (Hauger et al., 2003).

Expression of CRF₁ has been observed in frontal cortical areas, the cholinergic basal forebrain (medial septum and ventral and horizontal limbs of the diagonal band of Broca), the brainstem cholinergic nuclei (laterodorsal tegmental nucleus and the pedunculo-pontine tegmental nucleus), the ventral tegmental area, the superior colliculus, the basolateral nucleus of the amygdala (BLA), the cerebellum, the red nucleus, the trigeminal nuclei, the anterior pituitary (Steckler and Holsboer, 1999), the hippocampus, substantia nigra pars compacta and pars reticularis, the LC and at the level of the substantia innominata (Sauvage and Steckler, 2001) (Figure 2c).

The CRF₂ receptor has been cloned from man (Liaw et al., 1996; Valdenaire et al., 1997; Kostich et al., 1998), mouse (Perrin et al., 1995; Kishimoto et al., 1995; Stenzel et al., 1995), rat (Lovenberg et al., 1995b), frog (Dautzenberg et al., 1997), tree shrew (Dautzenberg et al., 1997; Palchaudhuri et al., 1999) and fish (Arai et al., 2001; Pohl et al., 2001).

Three functional splice variants of the CRF₂ receptor have so far been identified: CRF_{2(a)} (Lovenberg et al., 1995b), CRF_{2(b)} (Lovenberg et al., 1995b) and CRF_{2(c)} (Kostich et al., 1998).

CRF_{2(a)} is a 411 amino acid protein with approximately 71% identity to the CRF₁ receptor (Lovenberg et al., 1995b), and is the dominant CRF₂ splice variant expressed in the mammalian brain (Hauger et al., 2003).

CRF_{2(b)} is 431 amino acids in length and differs from CRF_{2(a)} in that the first 34 amino acids in the N-terminal extracellular domain are replaced by 54 different amino acids (Perrin et al., 1995). This receptor is primarily expressed in the periphery, and may be involved in some of the effects of CRF which are not centrally mediated.

Expression of the 397-amino acid CRF_{2(c)} receptor has only been detected in limbic regions of the human central nervous system, and it shows similar pharmacological characteristics to CRF_{2(a)} (Kostich et al., 1998).

CRF_{2(a)} is localised to subcortical regions, including the lateral septum, the PVN and VMH, the cortical and medial nuclei of the amygdala, and the serotonergic Raphé nuclei (Lovenberg et al., 1995b; Steckler and Holsboer, 1999) (Figure 2c).

Combined populations of both CRF₁ and CRF_{2(a)} have been reported in the olfactory bulb, the hippocampus, the entorhinal cortex, the BNST and the PAG (Lovenberg et al., 1995b)

CRF_{2(b)} is primarily localised to the heart, skeletal muscle, and in the brain to cerebral arterioles and choroid plexus (Lovenberg et al., 1995a). Due to this lack of expression in the brain, the CRF_{2(b)} receptor has not been implicated in stress related conditions, or the stress response.

The most recently described novel CRF₂ receptor is a truncated variant of CRF_{2(a)}, cloned from the rat amygdala (Miyata et al., 1999). Compared to the CRF_{2(a)} receptor, this new subtype is more strongly expressed in the rat

amygdala, thalamus and hypothalamus and it is suggested that this receptor may regulate endogenous CRF release in the amygdala (Miyata et al., 2001).

A role for CRF₁ in the regulation of HPA axis activity has been clearly demonstrated by studies using selective CRF₁ antagonists, such as CRA0450/R278995 (Chaki et al., 2004), CRA5626 (Steckler et al., 2006), CP-154, 526 (Schulz et al., 1996), antalarmin (Webster et al., 1996; Bornstein et al., 1998), SSR125543A (Gully et al., 2002), R121919 (Gutman et al., 2003), DMP696 (Maciag et al., 2002), DMP904 (Lelas et al., 2004) and CRA1000 (Pournajafi et al., 2001), which reduced CRF-induced increases in plasma ACTH and/or corticosterone. Indeed CRF₁ is the main CRF receptor subtype at the anterior pituitary level (Chalmers et al., 1995) and was therefore expected to have a strong influence on HPA axis activity (Steckler, 2001), while CRF_{2(a)} predominates at the level of the PVN (Chalmers et al., 1995).

Stress-induced corticosterone levels were reduced in CRF₁-deficient mice (Timpl et al., 1998). Basal ACTH secretion in these mice is normal, while stress-induced heightened ACTH levels are reduced (Timpl et al., 1998). Unaltered basal ACTH may be explained by the influence of other hormones stimulating ACTH release, such as vasopressin, which acts synergistically with CRF on ACTH release, but in contrast to CRF, is unable to increase ACTH synthesis under acute stress conditions (Aguilera et al., 1983; De Bold et al., 1984). In a second line of CRF₁-deficient mice (Smith et al., 1998), stress-induced ACTH and corticosterone responses were also reduced.

In addition to these two major CRF receptors expressed in the brain, a CRF binding protein (CRF-BP) exists. In the brain, CRF-BP is hypothesised to serve as a regulatory factor in HPA activation, as well as extrahypothalamic CRF neurotransmission (Kemp et al., 1998). Indeed, it has been shown to function as an endogenous buffer for CRF and related peptides (Behan et al., 1996). CRF-BP binds both native rat/human CRF and Urocortin 1 (discussed below) with higher affinity than CRF receptors (Vaughan et al., 1995). As Urocortin 1 also binds CRF-BP with high affinity, albeit less than CRF itself (Behan et al., 1996), it has been proposed that Urocortin 1 may act endogenously to elevate CRF levels through competition at CRF-BP.

A number of other peptides related to CRF and endogenous to various species have also been discovered. Some of these peptides have been used in the production of research tools (e.g. frog sauvagine (Erspamer et al., 1980) was used in the production of the selective CRF₂ antagonist antisauvagine-30 (Ruhmann et al., 1998)), and those endogenous to both rodents and humans have received particular interest. These peptides have been dubbed the Urocortins.

The Urocortins

Urocortin 1 (Vaughan et al., 1995) has 45% homology to human CRF (Table 1) and has a high affinity for both CRF₁ and CRF₂ receptors, greater than CRF itself, with approximately equal affinity for CRF₁ and CRF_{2(a)} (Table 2)

Mouse Urocortin 2 (Reyes et al., 2001) (mUrocortin 2) is a 38 amino acid peptide with 34% amino acid identity with rat CRF (Table 1) and is highly selective for CRF₂ (Table 2). Human Urocortin 2 (hUrocortin 2; also known as Stresscopin-related peptide) was originally identified along with hUrocortin 3 (also known as Stresscopin) from *Takifugu rubripes* (Japanese pufferfish), and *Tetraodon nigroviridis* (a freshwater pufferfish) (Hsu and Hsueh, 2001). hUrocortin 3 shares 32% amino acid identity with human CRF (Table 1), and hUrocortin 2 shares 34% homology to human CRF. Both hUrocortin 2 and hUrocortin 3 are highly selective for CRF₂, though hUrocortin 3 is less potent at CRF₂ than mUrocortin 3 (Lewis et al., 2001).

Table 1: Sequence comparison of CRF peptides. Identical amino acids in all peptides are underlined (Abbreviations: r, rat; h, human; o, ovine; m, mouse) (Adapted from Dautzenberg et al., 2001; Lewis et al., 2001)

Peptide	Amino acid sequence	Length	Homology (%)
rhCRF	SEPPIS <u>LD</u> LT <u>F</u> HLLREVLEMARAEQLAQQA <u>HS</u> <u>N</u> RKLMEII	41	100
oCRF	SQEPPI <u>SL</u> <u>D</u> LT <u>F</u> HLLREVLEMTKADQLAQQA <u>HS</u> <u>N</u> RKLLDIA	41	83
rUrocortin 1	DDPPL <u>S</u> <u>I</u> <u>D</u> LT <u>F</u> HLLRTLLELARTQSQRERAEQNRIIFDSV	40	45
hUrocortin 1	DNPSL <u>S</u> <u>I</u> <u>D</u> LT <u>F</u> HLLRTLLELARTQSQRERAEQNRIIFDSV	40	43
hUrocortin 2	IVL <u>S</u> <u>L</u> <u>D</u> VP <u>I</u> GLLQILLEQARARAAREQA <u>T</u> <u>T</u> <u>N</u> ARILARV	38	34
mUrocortin 2	VIL <u>S</u> <u>L</u> <u>D</u> VP <u>I</u> GLLRILLEQARYKAARNQA <u>A</u> <u>T</u> <u>N</u> AQILAHV	38	34
hUrocortin 3	FTL <u>S</u> <u>L</u> <u>D</u> VP <u>T</u> NIMNLLFNIAKAKNLRAQA <u>A</u> <u>A</u> <u>N</u> AHLMAQI	38	32
mUrocortin 3	FTL <u>S</u> <u>L</u> <u>D</u> VP <u>T</u> NIMNILFNIDKAKNLRAQA <u>A</u> <u>A</u> <u>N</u> AQLMAQI	38	26

Table 2: Inhibitory binding constants (K_i) (nM) of CRF peptides at human CRF receptors

Peptide	hCRF ₁	hCRF _{2(a)}	hCRF _{2(b)}	Reference
rhCRF	1.5	42	47	Dautzenberg et al., 2001
oCRF	1.1	230	320	
rUrocortin 1	0.3	0.4	0.4	
hUrocortin 1	0.4	0.3	0.5	
hUrocortin 2	>100	1.7	0.5	Lewis et al., 2001
mUrocortin 2	>100	2.1	0.66	
hUrocortin 3	>100	21.7	13.5	
mUrocortin 3	>100	5.0	1.8	

In the rat, Urocortin 1 expression has been found in the Edinger-Westphal nucleus, the lateral superior olive, the lateral hypothalamus and the supraoptic nucleus (all regions that have not been shown to express CRF mRNA) the cholinergic laterodorsal tegmental nucleus, the serotonergic dorsal raphe nucleus, and the PAG (Kozicz et al., 1998) (Figure 2d). Moderate levels of Urocortin 1 mRNA are also found in the hippocampus, the basal ganglia, the medial septum, the medial and cortical amygdaloid nuclei, the PVN and the VMH, the superior colliculus, the red nucleus, the substantia nigra, and the cerebellar cortex (Wong et al., 1996). A much more uniform distribution of

Urocortin 1 has been reported in the human brain, with Urocortin 1-like immunoreactivity in the frontal, temporal and occipital cortices, the thalamus, hypothalamus, pons, medulla oblongata and cerebellum (Takahashi et al., 1998). Direct comparison by Morin et al. (1999) of CRF and Urocortin 1 immunoreactivity in the rat brain revealed limited overlap of CRF and Urocortin 1 expression, only overlapping in the supraoptic nucleus and the hippocampus, potentially suggesting that the peptides have distinct functions.

Urocortin 2 mRNA expression is primarily subcortical, with major sites of expression in stress-related cell groups such as the PVN, supraoptic and arcuate nuclei of the hypothalamus, and the LC. Secondary sites of expression were observed in the motor nuclei of the brain stem (trigeminal, facial, hypoglossal), as well as in the spinal ventral horn (Reyes et al., 2001) (Figure 2d).

Urocortin 3 mRNA expression has been reported in the median preoptic nucleus, fornix, BNST, anterior and lateral hypothalamus and just lateral to the PVN, the dorsomedial hypothalamic nucleus, the ventral part of the anterior periventricular nucleus, the retrochiasmatic area, the medial amygdala and the auditory-related superior paraolivary nucleus (Lewis et al., 2001) (Figure 2d). Immunohistochemistry confirms this distribution (Li et al., 2002), and revealed Urocortin 3-immunoreactive fibres in the VMH, the predominant site of CRF₂ receptor (discussed below) expression within the hypothalamus (Chalmers et al., 1995; Van Pett et al., 2000), extending to the lateral arcuate nucleus, and from the rostral arcuate nucleus into the retrochiasmatic area. A small number of fibres were found in the dorsomedial nucleus and the lateral hypothalamus, while abundant fibres were present in the anterior hypothalamus. Fibres were also found in the medial preoptic area, and in the posterior hypothalamus. Urocortin 3-immunoreactive fibres extended through the supramammillary nucleus to the ventral premammillary nucleus. In the median eminence fibres were present in the internal zone. Outside the hypothalamus, fibres were present in the lateral septum, posterior BNST and medial amygdala, all CRF₂ expressing areas (Chalmers et al., 1995; Van Pett et al., 2000), and extended into the ventral hippocampus. In the thalamus, fibres were found in the posterior paraventricular nucleus and the lateral habenula. In the midbrain and brainstem, low numbers of fibres were found in areas with low CRF₂ expression, including the dorsal and

dorsolateral periaqueductal grey, the superior and inferior colliculi and the ventral lateral lemniscus (Li et al., 2002).

Based on the central distribution of the Urocortins and CRF₂ expressing neurons (discussed below), it has been suggested that Urocortin 1 may serve as the major endogenous CRF₂ ligand in the hindbrain, whereas Urocortin 3 may serve as the major CRF₂ ligand in the forebrain (Hauger et al., 2003). Urocortin 2, on the other hand, may signal at CRF₂ receptors expressed in regions lacking Urocortin 1 or Urocortin 3 innervation, e.g., hippocampus and certain regions of the cerebral cortex (Hauger et al., 2003).

CRF and its relationship with other systems

The distribution of the CRF-system components discussed so far overlaps with numerous regions and neuronal pathways associated with other neurotransmitter systems. In addition to such suggestive evidence for interactions with other neurotransmitter systems in the brain, many studies have provided further anatomical and functional evidence.

Glutamatergic System

Interaction between the CRF and glutamatergic systems appears to be both region and receptor dependent. Activation of CRF₁ receptors in the rat lateral septum with CRF facilitates glutamatergic synaptic transmission, while activation of CRF₂ receptors with Urocortin 1 depresses this transmission (Liu et al., 2004; Liu et al., 2005). Conversely, in the CeA, CRF depresses glutamatergic transmission, while Urocortin 1 facilitates it (Liu et al., 2004). Glutamatergic transmission in these nuclei appears to be tonically modulated through these receptors, as CRF receptor antagonists also modulated transmission (Liu et al., 2004).

Noradrenergic System

CRF projections originating in the CeA, the PVN and the BNST terminate in the noradrenergic LC, which may have a role in arousal in response to emotional stressors, and possibly affect attentional processes (Carli et al., 1983; Cole and Robbins, 1992; Usher et al., 1999). Noradrenergic projections return to the BNST and the CeA (Heinrichs et al., 1991; Koob and Heinrichs, 1999), raising the possibility of feedback loops.

Centrally administered (intracerebroventricular, or ICV) CRF, or CRF infused directly into the LC increases the firing rate of the LC (Curtis et al., 1997), and increases the release of noradrenaline in LC projections (Smagin et al., 1995). Infusion of CRF into the LC also produces an increase in catecholamine activity and turnover in the frontal cortex along with increased anxiety-related behaviour (Butler et al., 1990). ICV CRF and Urocortin 1 increased hippocampal

levels of noradrenaline and its metabolite 3-methoxy-4-hydroxyphenylglycol, whereas Urocortin 2 and Urocortin 3 elevated 3-methoxy-4-hydroxyphenylglycol, but not noradrenaline levels (de Groote et al., 2005). Activation of the LC by stress has also been prevented by administration of CRF antagonists directly into the LC region (Valentino et al., 1991; Curtis et al., 1994; Lechner et al., 1997). In addition, stressors increase the concentration of CRF in the LC (Chappell et al., 1986), and noradrenergic antagonists have been shown to reverse some of the stress-like effects of ICV CRF (Cole and Koob, 1988). Stress has also been shown to induce noradrenaline release in the PVN (Pacak et al., 1995b) and the BNST (Pacak et al., 1995a). Noradrenaline in the PVN and CeA may also be involved in stimulating the release of CRF (Raber et al., 1995).

A feed-forward system of interactions between the CRF and noradrenaline systems has been proposed (Koob, 1999), in which, after stimulation of the LC by CRF, as described above, noradrenaline is capable of stimulating CRF release in terminal projections of the forebrain noradrenergic systems, such as the PVN, the BNST and the CeA. It has been hypothesised that such a system may be an important link between activation of the HPA axis and the central nervous system, although such a system may also be vulnerable to dysfunction (Koob, 1999).

Serotonergic (5-Hydroxy-tryptamine; 5-HT) System

CRF interacts with the serotonergic system at the level of the dorsal raphe nuclei (DR) (Price et al., 1998), which provides one of the major sources for forebrain 5-HT innervation (Azmitia and Segal, 1978), and also affect a wide range of different types of behaviour (Soubrie, 1986; Steckler and Sahgal, 1995; Lucki, 1998). ICV injection of CRF or Urocortin 1, 2, or 3 has been shown to increase levels of 5-HT and its metabolite 5-HIAA in the hippocampus (Linthorst et al., 2002; de Groote et al., 2005), and the increase in hippocampal 5-HT associated with diving during forced-swim could be antagonised by the CRF antagonist D-Phe-CRF₁₂₋₄₁ (Linthorst et al., 2002). Mutant mice deficient for CRF₁ receptors have altered levels of 5-HIAA although not serotonin (Penalva et al., 2002). Further evidence supports effects of ICV CRF on 5-HT levels in other brain regions. ICV CRF has also been shown to influence 5-HT and 5-HIAA

levels in the DR and MR of previously stressed rats (Summers et al., 2003), and ICV CRF is reported to increase 5-HT and 5-HIAA levels in the dorsomedial hypothalamus (Lowry et al., 2001). Co-localisation of 5-HT and CRF receptors has been demonstrated in the DR (Day et al., 2004), and both ICV CRF, and CRF injected into the DR inhibit neuronal activity (although high doses may even increase activity) (Price et al., 1998; Kirby et al., 2000). The inhibitory effect of CRF in the DR could be blocked by CRF₁ antagonists (Kirby et al., 2000). However, in vitro, CRF has been reported to increase the firing rates of neurons from the ventral portion of the DR (Lowry et al., 2000). Injection of Urocortin 2 into the DR increased 5-HT efflux in the basolateral amygdala, a projection region of the DR, and increased c-fos expression in raphe serotonergic neurons, and these effects were blocked by the CRF₂ antagonist antisauvagine-30, suggesting that the effects are CRF₂-mediated (Amat et al., 2004). In the DR itself, low doses of Urocortin 2 actually appear to reduce the activity of serotonergic neurons through CRF₂, although at high doses of Urocortin 2, CRF₂-mediated deactivation of GABA-ergic neurons in the same region may lead to non-specific activation of the same serotonergic neurons (Pernar et al., 2004). Furthermore, chronic administration of either ovine CRF (which has a higher affinity for CRF₁ than CRF (Hauger et al., 2006)) or Urocortin 2 to the DR have been demonstrated to produce similar alterations in expression of genes associated with components of the serotonergic system in the DR (Clark et al., 2007). Injection of Urocortin 1 into the basolateral amygdala has been reported to increase the number of c-Fos-immunoreactive serotonergic neurons within subdivisions of both the dorsal raphe nucleus and median raphe nucleus (Spiga et al., 2006). Furthermore, ICV Urocortin 2 has been reported to increase c-Fos expression in serotonergic neurons of the DR (Staub et al., 2005; Staub et al., 2006). Overall, the evidence suggests a complex interaction between the CRF and 5-HT systems, perhaps more explicitly neuronal-dependent than CRF receptor-dependent. Such interactions may be of relevance to the pathophysiology underlying depression, and support a role for drugs altering activity of the CRF system in the (co)treatment of this disorder.

Dopaminergic System

In addition to their potential role in interactions between noradrenergic and CRF systems, CRF neurons in the CeA and BNST innervate dopaminergic neurons in the substantia nigra (Gray, 1993). Centrally administered CRF has been shown to increase concentrations of a dopamine metabolite in a number of brain regions (Kalivas et al., 1987; Dunn and Berridge, 1987), suggesting an increased drive for dopamine production. This is supported by in vitro evidence, as CRF has been reported to activate striatal tyrosine hydroxylase, the crucial enzyme in the production of dopamine (Olianas and Onali, 1988; Olianas and Onali, 1989). It has also been demonstrated that ICV CRF increases the activity of dopaminergic projections to the prefrontal cortex (Lavicky and Dunn, 1993). In a behavioural model believed to reflect stress- and anxiety-related states, the CRF-enhanced acoustic startle, a dopamine receptor antagonist has been demonstrated to inhibit the startle-enhancing effects of CRF (Meloni et al., 2006). In the same study, CRF neurons in the BNST, a region linked to the CRF-induced effects on startle (Lee and Davis, 1997), were found to be closely surrounded by dopaminergic neurons, providing anatomical evidence for interaction of these two systems.

Cholinergic System

A combination of evidence suggests intricate interactions between the CRF and cholinergic systems. Interaction between these two systems may be involved in cognitive function (see Warnock et al., 2006 for review).

Coexpression of elements of the CRF and cholinergic systems has been reported in cholinergic forebrain nuclei (medial septum, ventral and horizontal limbs of the diagonal band of Broca, substantia innominata, but not the nucleus basalis magnocellularis), and cholinergic brainstem nuclei (pendunculo pontine and laterodorsal tegmental nuclei) (Crawley et al., 1985; Austin et al., 1995; Sauvage and Steckler, 2001).

Intracerebroventricular (ICV) infusions of CRF have been reported to increase hippocampal acetylcholine (ACh) release (Day et al., 1998a; Day et al.,

1998b), an effect which is likely to be CRF₁-mediated (Gully et al., 2002). There is evidence that interactions between these two systems are reciprocal, with both CRF capable of affecting cholinergic activity and acetylcholine capable of modulating CRF activity (see Warnock et al., 2006 for review), as for example, chronic treatment with the non-selective muscarinic antagonist atropine produced an increase in CRF₁ in frontoparietal cortex in rats (De Souza and Battaglia, 1986), and acetylcholine induces CRF release from the amygdala in vitro (Raber et al., 1995).

Clearly there is a sizeable body of literature supporting interactions between the CRF system and a number of other neurotransmitter systems in the brain, with far-reaching implications in a range of behaviours and pathological states, including (e.g.) arousal/attention (noradrenaline/acetylcholine), reward/addiction (dopamine), anxiety/depression (serotonin). Furthermore, complex interactions exist between not only CRF and the systems discussed above, but also between those other neurotransmitters, with the consequence that the effects of CRF could be wide-reaching, and that endpoint responses could involve multiple neurotransmitter systems.

Implication of CRF in stress-related disorders

Alterations in CRF activity have been described in a range of neuroendocrine, neurological and psychiatric disorders, including major depressive disorder, post-traumatic stress disorder (PTSD), schizophrenia and dementia (Steckler, 2005). This makes the CRF system an interesting target for the development of novel treatments for these disorders.

CRF₁ antagonists are implicated in the potential treatment of PTSD, as studies have shown basal cerebrospinal fluid (CSF) CRF levels to be elevated in patients with the disorder, although low glucocorticoid levels have also been observed (see Kasckow et al., 2001 for a review). This surprising discrepancy has led to speculation of an adrenocortical insufficiency in these patients (Kasckow et al., 2001), supported by studies of cortisol secretion and glucocorticoid receptors (Yehuda et al., 1991a; Yehuda et al., 1991b; Yehuda et al., 1993; Yehuda et al., 1996). Neuroimaging studies have revealed functional impairment and shrinkage of the medial prefrontal cortex in PTSD (Rauch et al., 2003; Shin et al., 2005), a region which may be involved in negative feedback on the HPA axis (Diorio et al., 1993).

In depression, an increased number of CRF-immunoreactive neurons has been reported at the level of the PVN (Raadsheer et al., 1994), and in situ hybridisation revealed markedly elevated CRF mRNA levels in the PVN of depressed patients (Raadsheer et al., 1995). Chronic antidepressant treatment has been demonstrated to decrease CRF mRNA in the rat PVN (Brady et al., 1992), and decrease CRF₁ mRNA levels in the amygdala (Aubry et al., 1999), providing animal model support for these clinical findings. An increased CRF-like immunoreactivity in the cerebrospinal fluid of depressed patients has been reported in some studies (Banki et al., 1987; Nemeroff et al., 1988; Wong et al., 2000), which seems to decrease after successful antidepressant treatment (De Bellis et al., 1993; Heuser et al., 1998), while lack of normalisation of cerebrospinal fluid CRF levels during antidepressant treatment may predict early relapse (Banki et al., 1992).

Variations in the CRF-BP gene have been associated with major depression (Claes et al., 2003). A failure of CRF-BP levels to adapt to rising CRF levels, associated with depression (Mitchell, 1998), may contribute to increased “free” CRF levels and therefore the development of heightened stress responses or even psychopathology (Herringa et al., 2006a). Indeed, a recent post-mortem study of depressed suicide victims demonstrated increased CRF levels in the frontal cortex, whereas CRF-BP mRNA remained unchanged (Merali et al., 2004), and reductions in basolateral amygdala CRF-BP mRNA have been found in schizophrenic and bipolar patients (Herringa et al., 2006b), although the translation of these changes into protein levels was not confirmed.

Depressed patients have been reported to have a blunted ACTH response to exogenous CRF challenge (Gold et al., 1986; von Bardeleben and Holsboer, 1988) and to show an abnormal response to combined dexamethasone/CRF challenge, which has been reported in up to 80-90% of patients (Heuser et al., 1994). There is also evidence that these abnormalities can be normalised by antidepressant treatment, though differences in the onset of normalisation are apparent, and the recovery may only be temporary (Schule et al., 2006). In healthy volunteers, the HPA response to CRF challenge could be attenuated by antidepressant treatment (Michelson et al., 1997), and certain selective serotonin reuptake inhibitors (SSRIs) have been reported to reduce the activity of CRF neurons (Nemeroff and Owens, 2004). A decrease in CRF binding sites has been measured in the frontal cortex of suicide victims (Nemeroff et al., 1988), possibly secondary to elevated CRF levels (Steckler, 2005). In support of this, CRF has been demonstrated to downregulate CRF₁ binding in cortical areas of rats, although this was only after acute ICV administration (Brunson et al., 2002), and CRF₁ mRNA, but not CRF₂ mRNA has been found to be downregulated in the frontal cortex of depressed patients (Merali et al., 2004).

The precise reasons for HPA hyperactivity and, in particular, for enhanced production and release of CRF in depression remain unknown. It has been suggested that once the balance of corticosteroid receptor mediated events is disturbed, an individual loses the ability to maintain homeostasis if challenged, for example by an adverse life event, such as severe stress (De Kloet et al.,

1998). This leads to disturbed neuroendocrine regulation and impaired behavioural adaptation, which after a certain threshold can trigger the onset of a psychiatric disorder (Holsboer, 1999). Clinical studies support the theory of impaired glucocorticoid receptor function leading to insufficient cortisol-mediated negative feedback on CRF expression (Modell et al., 1997; Modell et al., 1998; Holsboer, 2000). Furthermore, in a rat model of social instability during adolescence, subsequent stress exposure induced abnormal HPA responses, both centrally and peripherally (increased plasma corticosterone and PVN CRF mRNA levels) (McCormick et al., 2007). The complex regulation of the HPA axis provides multiple chances for intervention in the event of such impairment (Holsboer, 1999).

It has been suggested that the suppression of CRF activity might be the final and common step of antidepressant action that is necessary for stable remission of major depressive disorder (Holsboer and Barden, 1996), and CRF receptor antagonists may provide a method to accelerate normalisation.

Local Cerebral Glucose Utilisation (LCGU)

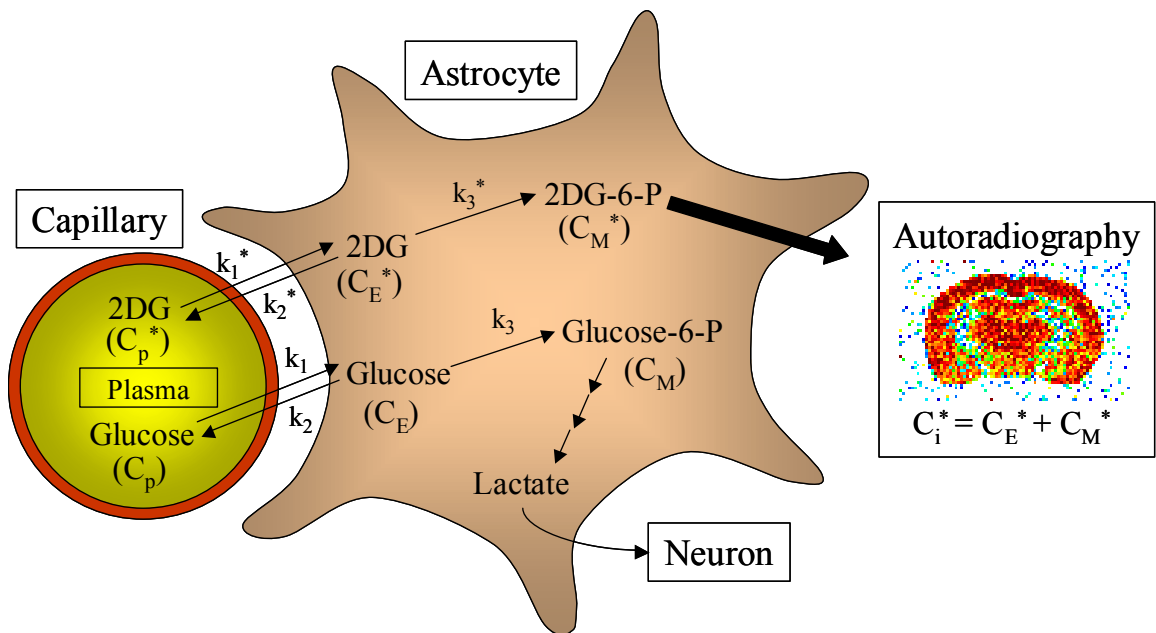
Glucose is the primary energy source for the mammalian brain, a continuous supply being necessary to maintain normal cerebral function (Maher et al., 1994). The development of the LCGU technique (Sokoloff et al., 1977) provided the demonstration for coupling between synaptic activity and glucose use. Thus, with a measure for glucose metabolism in regions of the brain, an estimate of synaptic activity in those regions can be obtained.

The LCGU technique is of particular interest due to its translational nature. Translational models are those techniques where the methodology and validity can be directly adapted between studies in animals and the clinic. As will be discussed here, the theoretical basis of the LCGU technique can be directly applied in humans, using positron emission tomography (PET).

Sokoloff's LCGU technique relies on the unique properties of the glucose analogue 2-deoxy-D-glucose (2-deoxyglucose; 2DG). This molecule differs from glucose by the replacement of the hydroxyl group on the second carbon atom with a hydrogen atom. 2DG is transported between blood and brain tissues by the same glucose transporters as glucose itself (GLUT1-5, GLUT8; (McEwen and Reagan, 2004)), and once in the tissues it competes with glucose for hexokinase, which phosphorylates both to their respective hexose-6-phosphates (Sols and Crane, 1954). At this point the metabolism of 2DG-6-phosphate (2DG-6-P) essentially ceases, as 2DG-6-P is not a substrate for the enzyme phosphohexoseisomerase, which converts glucose-6-phosphate to fructose-6-phosphate, allowing further metabolism via the glycolytic and tricarboxylic acid pathways (Sokoloff et al., 1977) (Figure 3). By radiolabelling the 2DG molecule its accumulation in brain tissues can be measured by conventional techniques, such as autoradiography or scintillation counting of homogenates. In the originally developed technique [^{14}C]-2DG was used, but since then [^3H]-2DG has also been made available, which along with improving safety considerations increases the resolution of the technique (Gallistel and Nichols, 1983), although the reduced specific activity of the latter radioligand could be considered a drawback.

Figure 3: Schematic illustration of the theoretical model (adapted from Sokoloff et al., 1977 and Magistretti and Pellerin, 1999b).

Glucose and 2DG are transported from the plasma into astrocytes by the same glucose transporters (k_1 and k_1^* represent the rate constants for transport from plasma to tissue, k_2 and k_2^* represent transport from tissue to plasma), once inside the cell they are converted to glucose-6-phosphate (glucose-6-P) and 2DG-6-phosphate (2DG-6-P), respectively, by hexokinase (rate constants k_3 and k_3^*). While 2DG-6-P is essentially trapped in the cell, glucose-6-P is metabolised further to lactate, which provides an energy source for neurons (see Magistretti and Pellerin, 1999b). C_P , C_E and C_M represent the concentrations of plasma glucose, intracellular glucose and glucose-6-P, respectively. C_P^* , C_E^* and C_M^* represent the concentrations of plasma 2DG, intracellular 2DG and 2DG-6-P, respectively. C_i^* , the total tissue radiolabel concentration is the combination of C_E^* and C_M^* . These terms are used in the operational equation of the LCGU technique, discussed later.



In order to calculate the rate of local cerebral glucose metabolism, Sokoloff et al. constructed the model:

$$R_i = \frac{C_i^*(T) - k_1^* e^{-(k_2^* + k_3^*)T} \int_0^T C_p^* e^{(k_2^* + k_3^*)t} dt}{\left(\frac{\lambda V_m^* K_m^*}{\Phi V_m K_m^*} \right) \left(\int_0^T (C_p^*/C_p) dt - e^{-(k_2^* + k_3^*)T} \int_0^T (C_p^*/C_p) e^{(k_2^* + k_3^*)t} dt \right)}$$

Where R_i is the rate of LCGU in a given region (in $\mu\text{mol}/100\text{g}/\text{min}$), k_1 , k_1^* , k_2 , k_2^* , k_3 , k_3^* , C_P , C_E , C_M , C_P^* , C_E^* , C_M^* , C_i^* represent the parameters detailed above, T is the time after which the experiment was ended (0 is zero time), λ is the ratio of the distribution space of 2DG in the tissue to glucose, Φ is the fraction of glucose that once phosphorylated continues down the glycolytic pathway, and K_m^* , V_m^* , V_m , K_m , represent the Michaelis Menten pharmacokinetic constants of hexokinase for 2DG and glucose, respectively.

This can be generalised to:

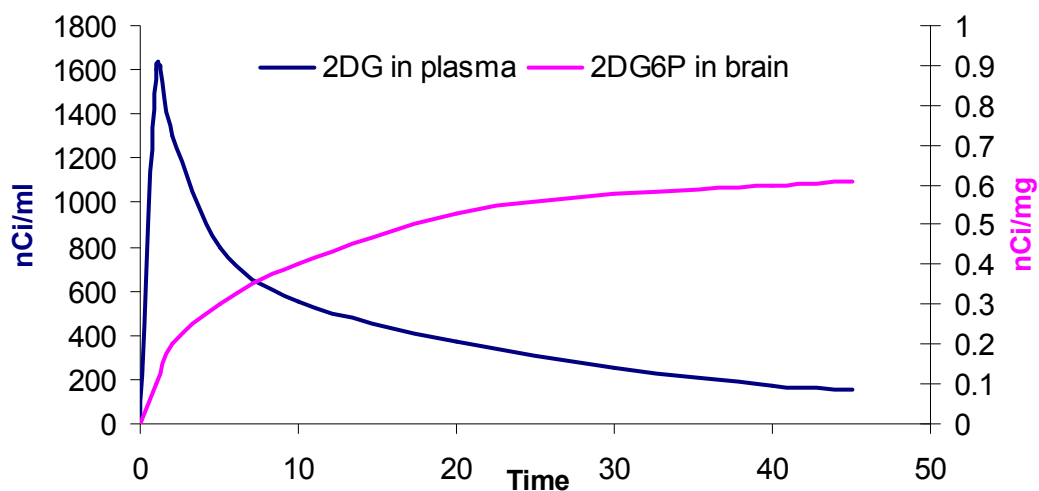
$$R_i = \frac{\text{Labeled product formed in interval of time, 0 to T}}{\left(\text{Isotope effect correction factor} \right) \left(\text{Integrated precursor specific activity in tissue} \right)}$$

Labeled product formed in interval of time, 0 to T
 Total label in tissue at time T - Label in precursor remaining in tissue at time T
 Integrated precursor specific activity in tissue
 Integrated plasma specific activity - Correction for lag in tissue equilibration with plasma

The calculation of actual molar LCGU values (in $\mu\text{mol}/100\text{g}/\text{min}$) using this equation requires repeated (rapid in the early phase of the protocol) arterial blood sampling for the measurement of plasma 2DG and blood glucose levels, which represents one of the drawbacks of the originally designed technique, as

this blood sampling is both labour intensive and risks disturbance of the animal. Particularly when studying stress-related effects or the CRF system, disturbance of the subject is problematic as this represents a mild stressor in itself, with knock on effects on the HPA axis and possibly on central responses. The expected plasma curve of 2DG after intravenous (IV) administration is illustrated in Figure 4. The rapid repeated sampling in the protocol is necessary to measure the rapid peak in plasma 2DG. Also illustrated is the expected cumulative curve of 2DG-6-P in the brain. Blood glucose (not illustrated) would in an ideal situation be a constant of approximately 150 mg/dl.

Figure 4: Illustrative figure of plasma and brain levels of 2DG and 2DG-6-P, respectively, after an IV injection of radiolabelled 2DG (adapted from Sokoloff et al., 1977)



Cellular mechanisms of LCGU

Measuring LCGU using 2DG does not reflect synaptic activity directly, but measures activity-dependent energy consumption (Magistretti and Pellerin, 1999b). It has been suggested that astrocytes play a central role in the distribution of energy substrates from the circulation to the neurons (Golgi, 1886; Sala, 1891; Andriezen, 1893), and the entire surface of central capillaries is covered by astrocytic end-feet (Peters et al., 1991). Glucose transporters of the GLUT1 type are expressed on these astrocytic end-feet (Morgello et al., 1995).

Using cortical glutamate as an example, the drive of glucose metabolism by neurotransmission can be illustrated.

Glutamate released during neurotransmission accumulates in the synaptic cleft, from where it is taken up by glutamate transporters in astrocytes, driven by an ATP-dependent Na⁺/K⁺-ATPase mechanism (Magistretti and Pellerin, 1999b). Glutamate stimulates the glycolytic processing of glucose in astrocytes, as indicated by increased lactate release and 2DG uptake (Pellerin and Magistretti, 1994).

It is suggested that astrocytes preferentially process glucose glycolytically into lactate, which once transported into neurons by specific lactate transporters, can be transformed by neurons into pyruvate and enter the tricarboxylic acid cycle, yielding 17 ATP molecules (Magistretti and Pellerin, 1999b). Indeed, *in vivo*, electrical stimulation resulting in neuronal activation has been associated with increased lactate levels (Hu and Wilson, 1997).

Modifications to the technique

While Sokoloff et al's original technique generates a fully quantitative measure of LCGU via the collection of repeated data points during the protocol, simpler versions of the technique have also been used in the literature to provide estimates of LCGU. These modifications typically do not measure plasma 2DG or glucose curves, meaning that actual molar LCGU cannot be calculated. However, the use of appropriate control groups and methods for standardising the protocol maintain the reliability of the technique.

In the original technique described by Sokoloff et al., the animal was placed in a hind-limb plaster cast to facilitate blood sampling, and LCGU was measured either in the anaesthetised state or conscious. However, with the aim of studying LCGU related to behaviours associated with CRF and stress, for example anxiety-related behaviour, it is clearly preferable to measure LCGU in the conscious, normally behaving animal. In addition to limiting normal behaviour, a hind-limb plaster cast can be compared to half-restraint/immobilisation stress, which itself is associated with behavioural effects

similar to CRF administration, and activates the HPA axis (McBlane and Handley, 1994; Jamieson et al., 2006; Rivalland et al., 2007).

In response to these drawbacks, the method was adapted for freely moving rats by Crane & Porrino (Crane and Porrino, 1989), who externalised the arterial and venous catheters via the nape of the neck, allowing easy access by the experimenter (used, for example, in Sarter et al., 1989). These catheters can be connected to a fluid swivel allowing remote infusion and blood sampling while maintaining free motion. However, it should be noted that any surgical intervention needs to be followed by a suitable period for recovery to an unstressed level, during which there is a risk of losing catheter patency through clotting. Some studies have measured LCGU within hours of surgery, at which point LCGU may be affected by the after-effects of prolonged anaesthesia or the physical stress associated with surgery and post-operative pain.

The technique has also been adapted to remove the need for surgically implanted catheters altogether. The function of 2DG as a tracer persists whether it is injected intravenously or via another route. Both intraperitoneal and subcutaneous administration have been used in the literature (Meibach et al., 1980; Kelly and McCulloch, 1983b; Kelly et al., 2002), and estimated LCGU after intraperitoneal injection of 2DG was found to highly correlate with that after intravenous injection (Meibach et al., 1980). While a fully calculated measure of LCGU ($\mu\text{mol}/100\text{g}/\text{min}$) is lost in the absence of continuous blood sampling, in the absence of catheters the animal can be entirely freely moving, remain in its home-cage between treatments and generally be less disturbed in the course of the protocol, a significant benefit.

The effect of fluctuating blood glucose is not accounted for in protocols without continuous blood sampling. In the original technique it is a requirement that blood glucose levels be constant during the protocol. Later, Sokoloff et al (1983) reported a modified operational equation which could account for changing blood glucose. However, it has also been demonstrated that similar patterns of LCGU can be obtained in the absence of repeated blood glucose and plasma 2DG measurement, although these representative measurements of LCGU cannot be expressed in $\mu\text{mol}/100\text{g}/\text{min}$ (Meibach et al., 1980).

When using a modification of the technique which is not fully corrected for blood glucose and 2DG, efforts have been made to standardise the results in another manner. One reported modification measures the ratio of radioactivity in grey matter:radioactivity in white matter, i.e. nCi/mg grey matter:nCi/mg corpus callosum (Kelly and McCulloch, 1983a; Mitchell and Crossman, 1984). This modification assumes that LCGU is unaltered in white matter, but there is evidence that this may not be the case (Kennedy et al., 1982), meaning that effects may be masked or accentuated by calculating such a ratio.

Many studies of LCGU use a single dose of 2DG (e.g. 50 μ Ci [14 C]-2DG – Sokoloff et al., 1977) in all animals, regardless of bodyweight, relying instead on the equation or ratio calculation to standardise their results. However, it could be argued that in studies using inbred rodent strains, with standardised, freely available diet, of matching age/bodyweight, that using a per kilogram dose of 2DG already provides a standardised experimental environment. Furthermore, in studies lacking continuous blood sampling, it remains possible to measure blood glucose and residual 2DG in the plasma upon sacrifice, and thus ensure that treatments have not produced significant changes in either measure, which could have knock-on effects on LCGU.

Both autoradiography and brain dissection have been used in studies of LCGU, and each has pros and cons. Autoradiography can be a time-consuming process, requiring sectioning, exposure to film and densitometry to generate data. Using 14 C-2DG, imaging requires approximately 5 days, yet does not have the resolution of 3 H-2DG (Gallistel and Nichols, 1983), which requires 3-4 weeks for imaging. Furthermore, the availability of a cryostat for sectioning and a computerised system for densitometry can be limited, while the dissection of regions of interest from the brain and measurement of LCGU in homogenates requires a separate set of instruments, which may be more readily available, and dependent on the number of samples can generate data more rapidly than autoradiography. However, dissection has a number of clear drawbacks compared to autoradiography. Heterogeneous regions such as the thalamus are grouped into one dissected region, making it more difficult to draw clear conclusions from alterations in LCGU in such regions. Indeed, dissection of

these regions may effectively mask changes in LCGU. For example, simultaneous increased LCGU in a particular subregion, with reduced LCGU in another subregion, grouped by dissection, could be missed through averaging to an overall unchanged response. The use of dissection also greatly reduces, and in many cases removes, the ability to measure LCGU in small brain regions. This effect can be twofold, as while a region may be too small to accurately dissect, the amount of radiolabelled 2DG in a small but dissectable region may be too low to accurately count.

Another drawback of techniques to measure LCGU is a lack of cellular resolution, or even regional resolution with coarse techniques such as dissection. Standard autoradiography, or dissection, allows only regional resolution, and that determined also by the size of the region. Sokoloff et al reported their ^{14}C -2DG method to have at best a resolution of 200 μm (Sokoloff et al., 1983).

Studies of 2DG uptake at the cellular level have shown that for most brain regions, the accumulation of radioactivity per unit area of neuropil and neuronal cell bodies is similar (Duncan et al., 1987; Duncan et al., 1990; Duncan and Stumpf, 1991). Therefore, at the regional topographic level, the vast majority of 2DG uptake observed in autoradiograms reflects the uptake of the compound in nerve terminals and other neuronal and glial processes that compose neuropil (Duncan et al., 1993).

Itoh et al (Itoh et al., 2004) have brought cellular resolution of LCGU one step closer with their development of a fluorescent 2DG analogue, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG). Uptake of the ligand was demonstrated both in vitro, and in vivo with accumulation of phosphorylated 2-NBDG in hippocampal neurons and cerebellar Purkinje cells.

Translation of preclinical measurements of LCGU to the clinic was made possible by the development of a PET (positron emission tomography) ligand - ^{18}F Fluoro-2-Deoxyglucose (FDG) (Reivich et al., 1979), which was successfully used to measure LCGU in humans (Phelps et al., 1979; Reivich et al., 1979). However, the resolution of this technique is still less than that of detailed autoradiography, although constantly improving, with current equipment reaching

a spatial resolution of 1.3 mm (microPET Focus, Concorde Microsystems Inc., Knoxville, TN, USA; Larobina et al., 2006).

Recently, an in vivo microprobe capable of locally measuring the time-concentration curve of a radiolabel has been developed. The “betaP” (Millet et al., 2004) is an alternative to ^{18}F FDG-PET, capable of acquiring time-concentration curves with high temporal and spatial resolution (detection with ^{18}F FDG is possible within an 0.8 mm^3 area around the probe).

To summarise, it can be argued that methods for measuring LCGU without full calculation do not account for individual subject variability in as much detail as the full technique, but these so-called “semi-quantitative” techniques can introduce more flexibility to experimental design.

LCGU and Cerebral Blood Flow

Close links between LCGU and regional cerebral blood flow (rCBF) have been demonstrated, with matching effects seen in some studies (Kelly and McCulloch, 1983c; Takamatsu et al., 2003a). It has long been postulated that increased neuronal energy metabolism is driven by increased cerebral blood flow and vice versa (Magistretti and Pellerin, 1999b), and in addition to evidence that neuronal glucose supply is regulated by astrocytes (Magistretti and Pellerin, 1999a), evidence has been found linking astrocytes and rapid changes in rCBF (Takano et al., 2006). However, LCGU and rCBF may not always be directly linked, as there is also evidence for the uncoupling of these two measures (Magistretti and Pellerin, 1999a). The muscarinic receptor antagonist scopolamine has been shown to inhibit rCBF in response to somatosensory stimulation in rats without affecting LCGU (Villringer and Dirnagl, 1995), and conversely, electrical stimulation of the nucleus basalis produced increased rCBF without affecting LCGU (Vaucher et al., 1997). Thus, in some cases, the study of neuronal activity using both LCGU and rCBF may provide a replication of data, though that may not always be the case.

LCGU and stress-related disorders

The value of measuring LCGU in the clinic can be considered two-fold. Not only can LCGU be used as a technique to investigate the structures of the brain that are involved in stress-related disorders, but the technique can also be used in the progression of novel drug candidates. In this latter function, the LCGU technique can be used in two ways. As an independent clinical study, the technique can be used to demonstrate the penetration of a drug into the human CNS in a manner sufficient to alter CNS activity. Secondly, the translational nature of the technique allows cross-species replication of drug-induced effects, hopefully increasing that drug's value as a candidate for use in patients. Altered LCGU in particular brain regions in patients can be associated with particular pathology or successful drug treatment, guiding research in animals.

Altered LCGU has been shown in depressed patients (Pizzagalli et al., 2003; Yuuki et al., 2005), along with HPA axis dysfunction (Aihara et al., 2007), and evidence has suggested that successful treatment may normalise these changes (Navarro et al., 2004; Aihara et al., 2007). The antidepressant fluoxetine has been reported to reduce LCGU in the amygdala, hippocampus and ventral striatum, all areas implicated in stress-related pathways, and to increase LCGU in the right superior parietal lobe (Cook, Jr. et al., 1994; Freo et al., 2000). Following tryptophan depletion, patients with major depressive disorder had increased LCGU in the orbitofrontal cortex, medial thalamus, anterior and posterior cingulate cortices, and ventral striatum compared to sham-depleted and normal controls (Neumeister et al., 2004), suggesting a serotonin-related trait dysfunction. Increased LCGU has also been reported in areas of frontal, cingulate, insula, and temporal cortex in association with different components of the severity and course of illness in treatment-resistant unipolar depression (Kimbrell et al., 2002).

Altered LCGU and regional cerebral blood flow has also been reported in panic disorder (Reiman et al., 1984; Nordahl et al., 1990; Reiman, 1997; Nordahl et al., 1998; Bisaga et al., 1998; Sakai et al., 2005), and LCGU was reduced in imipramine-treated panic disorder patients compared to unmedicated patients (Nordahl et al., 1990). State LCGU is increased in specific brain regions in

patients with panic disorder. Sakai et al (2005) found heightened LCGU by FDG-PET in the bilateral amygdala, hippocampus, and thalamus, and in the midbrain, caudal pons, medulla, and cerebellum compared to normal controls, implicating the amygdala-based fear network in panic disorder, and based on the expression of CRF and CRF receptors in this region, providing a possible link to CRF. Altered LCGU in the hippocampus region has been reported after administration of the anxiolytic drugs ipsapirone and buspirone (Wree et al., 1987; Grasby et al., 1992; Grasby et al., 1993) implicating the serotonergic system. The septo-hippocampal system has been linked to anxiety as part of a behavioural inhibition system (Gray, 1983; comprehensively discussed in Gray and McNaughton, 2000).

In addition to being implicated in panic disorder, CRF is likely to be involved in the amygdala-based fear network. After “priming” (sensitisation by repeated treatment) of the basolateral amygdala by CRF or Urocortin 1 infusion, Sajdyk et al (Sajdyk et al., 1999) demonstrated behavioural and cardiovascular responses to the human panicogenic agent sodium lactate. Furthermore, Choi et al (2005) found increased CRF-like immunoreactivity and CRF mRNA in the central nucleus of the amygdala after administration of doxapram, which causes panic anxiety in humans. Moreover, in addition to reduced fear-like behaviour, rhesus monkeys with bilateral lesions of the central amygdala exhibit reduced CRF levels in the CSF (Kalin et al., 2004). Increased LCGU in the amygdala, among other regions, of panic disorder patients implicates the amygdala-based fear network (Sakai et al., 2005). Patterns of altered LCGU after treatment with compounds acting on benzodiazepine receptors further support the involvement of the amygdala and other limbic regions in anxiety-related behaviour. Diazepam reduced LCGU in a range of regions including the amygdala, hippocampus and frontal cortex (Ableitner et al., 1985; Eintrei et al., 1999). In contrast, treatment with the anxiogenic benzodiazepine partial inverse agonist FG7142 (Corda et al., 1983; File and Pellow, 1984) has been reported to increase LCGU in limbic regions (Ableitner and Herz, 1987), although the amygdala was not studied in detail in this study.

Such detectable and reversible alterations in LCGU in stress-related disorders suggest that it is a useful paradigm with which to further investigate the underlying pathology in these disorders.

Using LCGU to Study the CRF system

Although the anatomy of the CRF system has been studied in detail, and the distribution of its components documented, it is particularly difficult to study as a whole. There are numerous studies using manipulation of the CRF system to study brain areas involved. These can involve lesioning, administration into specific brain regions and microdialysis. However, these studies invariably only study at most a few regions concurrently, meaning that effects in other regions might be missed. An advantage of the LCGU technique is that following a manipulation, whether pharmacological or behavioural (e.g. a stressor), the number of brain regions in which LCGU can be measured is limited only by the resolution of the technique, thus allowing global analysis of effects in the brain. What the technique does not allow is the chronological sequence of any response to be measured, as at earlier time points in the protocol a combination of unmetabolised and metabolised 2DG is present.

By comparing the effects of targeting either CRF₁ or CRF_{2(a)} receptors in the brain, it may be possible to further elucidate the roles of the two receptors. Furthermore, effects on LCGU can be compared to other measures of brain activity, such as increased expression of the immediate-early gene c-fos, which is rapidly induced in specific neuronal populations after stress, sensory stimuli, pain and pharmacological manipulation (Hughes and Dragunow, 1995), although the measurement of LCGU adds the possibility to measure reduced activity in a given region, not possible with c-fos.

The effects of CRF itself on LCGU have been studied, revealing that CRF consistently induces increases in LCGU in a wide range of regions in the rodent brain. Sharkey et al (1989) reported wide-spread alterations in LCGU following ICV CRF administration, including increases in frontal cortex, thalamic areas, the lateral hypothalamus and median eminence, the cerebellum, midbrain regions including the median raphe and locus coeruleus, regions of the medulla oblongata and in the fornix, which may relate to its connections between hypothalamus and hippocampus (Sharkey et al., 1989). Interestingly, simultaneous reductions in LCGU were seen in an area of the prefrontal cortex and the dorsal tegmentum in the same study. In developing rats similar

increases in LCGU have been reported in response to central CRF administration, and matched to increases in c-fos mRNA expression (Dube et al., 2000). Additional increases in LCGU were reported in the amygdala and hippocampus, and overall prefrontal cortex LCGU was in this case increased. Regions exhibiting increased c-fos mRNA expression matched those with increased LCGU, with additional increases seen in the PVN and anterior hypothalamus. Data reporting that the consistent increases in LCGU in these wide-spread regions are dose-dependent has been provided (Freo et al., 2005).

Studies of the effect of CRF on c-fos expression reveal a pattern of activation similar to that seen on LCGU, and indeed similar to that following stress. Centrally administered CRF has been reported to induce c-fos mRNA expression in limbic structures, including the lateral septum, hippocampus, amygdala, hypothalamus (notably the PVN), and in regions of the thalamus and brainstem (notably the LC) (Imaki et al., 1993). Increased c-fos immunoreactivity has also been reported in a number of these brain regions after either intravenous or intraperitoneal administration of CRF or Urocortin 1, including the PVN, LC and central amygdala (Wang et al., 2000; Maillot et al., 2003). Urocortin 2 has been demonstrated to increase c-fos expression in serotonergic neurons in the dorsal raphe (DR) after either ICV or direct-DR injection (Amat et al., 2004; Staub et al., 2005). C-fos activation after ICV Urocortin 2 administration was reported in the bed nucleus of the stria terminalis, central amygdala, PVN, parabrachial nucleus and nucleus of the solitary tract (Reyes et al., 2001). Notably, there is overlap in the c-fos expression patterns induced by these different peptides, suggesting that there may be overlapping function of either peptide or receptor in certain regions.

The different and complementary information obtained by assessment of c-fos activity and LCGU illustrates the utility of applying both functional mapping approaches to examine neuroanatomical correlates of behavioural states and drug treatment (Duncan et al., 1993).

Outline and Scope of this Thesis

Based on the differing distributions of the endogenous CRF-related peptides, CRF, Urocortin 1, 2, and 3 in the rodent brain, their differing affinity for central CRF receptors, and differing effects of these peptides after acute central administration in a range of stress-related paradigms, it was hypothesised that these peptides influence neuronal activity in different subsets of rodent brain regions.

The LCGU paradigm developed by Sokoloff et al. (1977) was chosen for its ability to simultaneously measure neuronal activity in numerous brain regions after physical or pharmacological manipulation. A number of modifications to this method have been reported in the literature, and therefore an initial aim of this thesis was to assess the usefulness of these modifications and select those which would be used further to study the effects of the endogenous CRF-related peptides.

After the selection of a suitable version of the LCGU paradigm, the primary aim of this thesis was to examine the patterns of neuronal activation produced in the rodent brain after acute administration of the CRF-related peptides, and to study the involvement of either or both CRF₁ and CRF₂ receptors in the LCGU response, using available selective antagonists and mutant mice lacking a functional CRF₁ receptor.

In order to compare the measured LCGU responses to a measure of the stress response, it was decided to combine LCGU measurement with measurement of plasma corticosterone to provide an indication of HPA axis activation.

Finally, in order to compare the effects of the CRF-related peptides on LCGU with a stressor, the effect of restraint stress on LCGU was studied, also in combination with measurement of plasma corticosterone, and the involvement of the CRF₁ receptor in the response investigated using a selective antagonist.

Chapter 2 – Assessment of the LCGU Protocol and Modifications

Assessment of the LCGU protocol and modifications

Introduction/Aims

Prior to using the LCGU technique to study experimental manipulations, it was necessary to select and assess modifications to Sokoloff et al.'s original technique which may be beneficial.

These modifications include: the use of ^3H -2DG in the place of ^{14}C -2DG, major benefits of which include greatly reduced cost and increased safety; “semi-quantitative” modifications such as calculating grey:white matter ratios or dissecting brain regions rather than autoradiography, which can remove the need for rapid blood sampling, reducing disturbance to the animal; and alternative routes of 2DG administration, as intravenous administration involves either surgical intervention or a relatively stressful injection procedure.

In addition to the LCGU techniques, a method for central (intracerebroventricular; ICV) administration of peptides was assessed. This method removes the need for surgical implantation of cannulae, while maintaining an accurate method for the administration of compounds which cannot cross the blood-brain barrier.

“In-house” assessment of the technique and its modifications allowed the selection of a final technique which provided a balance between efficiency and experimental power/reliability for further use. In addition, the process allowed hands-on assessment of the pros and cons associated with the technique, and provided multiple methods of measuring LCGU which could later be used to replicate important results.

Study 1 - Fully Quantitative Measurement of LCGU with ^3H -2DG and Phosphoimaging

Introduction

The use of ^3H -2DG for the measurement of LCGU can greatly reduce the experimental cost. In addition, this radioisotope is safer than ^{14}C . LCGU measured using ^3H -2DG has been demonstrated to produce results highly correlated to those using ^{14}C -2DG, and furthermore, ^3H -2DG may provide higher resolution on autoradiographs under the right conditions, although imaging requires substantially longer (Meibach et al., 1980).

Fully quantitative measurement of LCGU was achieved by taking a full time course (10 seconds to 45 minutes after 2DG infusion) of blood samples via surgically implanted arterial and venous catheters. Measurement of blood glucose, plasma 2DG and final brain 2DG content allowed calculation of LCGU using Sokoloff et al.'s operational equation.

An additional modification validated in this study was the use of a phosphoimager in the place of traditional autoradiography. Instead of film, the Fujix BAS 2000 Phosphoimager uses storage phosphor plates. These plates store an image of the radioactive content in sections of the brain, which can then be scanned. This process can be completed for ^3H -radioligands in approximately 3 days, compared to 4-6 weeks for autoradiography.

In order to validate the use of ^3H -2DG and the phosphoimager for the LCGU technique in our laboratories, a pilot study using three rats was performed, to allow comparison to results in the literature.

Methods

Subjects

Three male Sprague-Dawley rats (Charles River Laboratories) were individually housed in individually-ventilated cages with food and water available ad libitum, under a standard 12h/12h light-dark cycle (lights on 06:00 a.m.), and at a regulated temperature of $22 \pm 0.5^{\circ}\text{C}$ and humidity of $50 \pm 3\%$ for at least 1 week prior to surgery. Approximate weight at surgery was 250g. Following surgery, rats were housed in standard cages in an automated blood-sampling apparatus (Accusampler, DiLab, Sweden), under the same light conditions and food and water still available ad libitum. Final testing was carried out between 0700 and 1200 hours. All testing was conducted according to the European Communities Council Directive Nov. 1986 (86/609/EEC) and approved by the animal care and use committee of Johnson & Johnson Pharmaceutical Research and Development.

Surgery

Rats were anaesthetised using a mixture of 37.5 mg/kg ketamine (Ketalar, Parke-Davis, Belgium) and 0.25 mg/kg metedomidine (Domitor, Pfizer, Belgium) at a dose of 1ml/kg injected intraperitoneally (IP), which was sufficient for approximately 2h of surgery. Where necessary, small amounts of the anaesthetic mixture were subsequently administered IP to maintain a surgical level of anaesthesia.

Once fully anaesthetised, an antibiotic was administered (enrofloxacin 40mg/kg SC; Baytril, Bayer, Belgium) before surgery commenced. The hair was shaved on the belly, left groin area, between the shoulder blades and on the throat.

Implantation of arterial/venous catheters

An incision was made in the skin of the throat to allow blunt dissection of the right jugular vein and carotid artery, and a second incision was made in the skin between the shoulder blades. Using a trocar two polyurethane catheters

were routed subcutaneously from the dorsal incision to the ventral incision. These catheters were filled with a heparinized saline solution and implanted in the artery and vein, then temporarily plugged. The throat incision was closed with absorbable sutures, and a stainless steel coil tether was attached by means of a Dacron mesh button sutured with thick gauge non-absorbable sutures between the shoulder blades to protect the catheters. The dorsal skin incision was then closed with non-absorbable sutures.

Finally, a post-operative analgesic (buprenorphine 0.5mg/kg SC; Temgesic, Schering-Plough, Belgium) and an anaesthesia reversing agent (atipamezole 1 mg/kg SC; Antisedan, Pfizer, Belgium) were injected.

Following surgery the catheters were connected to continuous infusion pumps via a mobile swivel (Instech Laboratories Inc., USA), allowing the animal to move freely within its home-cage. To maintain the patency of the catheters a lightly heparinized saline solution was infused continuously during the week of recovery.

Drugs

2-deoxy-D-[1-³H]-glucose (1.0 mCi/ml, specific activity 14.0Ci/mmol) was purchased from Amersham, and administered IV at a dose of 300 μ Ci/kg.

Blood Sampling

Arterial blood samples were taken manually using a syringe and PE tubing from above the swivel at time points $t = 0.25, 0.5, 0.75, 1, 2, 3, 5, 7.5, 10, 15, 25, 35, 45$ minutes. Excess blood was drawn into the tubing for sampling in order to exclude dead-volume blood (approx. 70 μ l in the catheters used), and a sample of between 100-150 μ l taken into tubes containing EDTA to prevent clotting. The dead-volume blood and saline to replace the volume taken was returned to the animal after each sample.

Blood Analysis

10µl of whole blood from each sample was tested for glucose content using a commercially available blood glucose meter (Glucotouch ®, Lifescan, USA). The samples were then centrifuged (1100g, 4°C, 5 min) to separate the plasma. 20µl plasma was taken for scintillation counting to determine ^3H content.

Tissue Removal

After the final blood sample was taken (experimental time 45 minutes) the animals were sacrificed using carbon dioxide gas, and the brain removed. Brains were frozen in isopentane chilled to approx. -35°C on dry ice, and stored at -80°C until cutting of sections on a cryostat.

Authoradiography

20µm brain sections were cut on a Leica cryostat at -20°C , and thaw mounted on glass slides, before being dried at room temperature. The slides were then exposed for 3 days to Fujifilm BAS TR imaging plates with standards. The plates were scanned on a Fujix BAS 2000 Phosphoimager with a resolution of 100µm, and images analysed using AIDA 2D densitometry software (Raytest, Germany). Regions of interest were drawn and annotated by hand, and background subtraction was performed on a section of image not exposed to brain sections.

Calculations & Statistics

With the assistance of Johnson & Johnson Pharmaceutical Research & Development's biostatisticians, measured values for plasma ^3H , blood glucose and tissue ^3H were converted into LCGU values using Sokoloff et al.'s operational equation. Calculated values were compared to appropriate literature values using Spearman's Rank correlation coefficient in Graphpad Prism 5.1.

Results

A sample image obtained in the present study is shown in Figure 5. The lowest LCGU was measured in white-matter areas, namely the corpus callosum and internal capsule (see Table 3), and the highest in the auditory cortex and inferior colliculus.

The data correlated highly with the literature ($R_s = 0.87$, 95% confidence interval 0.6887-0.9493, $p < 0.0001$, against control values from Sokoloff et al., (1977) (N.B. actual LCGU values appear higher in this study due to the higher specific activity of ^{14}C -2DG); $R_s = 0.90$, confidence interval 0.7559-0.9615, $p < 0.0001$, against control values from Sarter (1990), in which ^3H -2DG was also used). Correlation graphs for the present data against these two cited sources are shown in Figure 6 (a) and (b). Comparative values from these studies are displayed in Table 3.

The measured distribution of ^3H -2DG in the plasma and blood glucose levels used in the calculation of LCGU are shown in Figure 7 and Figure 8).

Figure 5: Sample image obtained from the BAS 2000 phosphoimager for a rat injected with 2DG. Calibrated standards can be seen in the upper left corner.

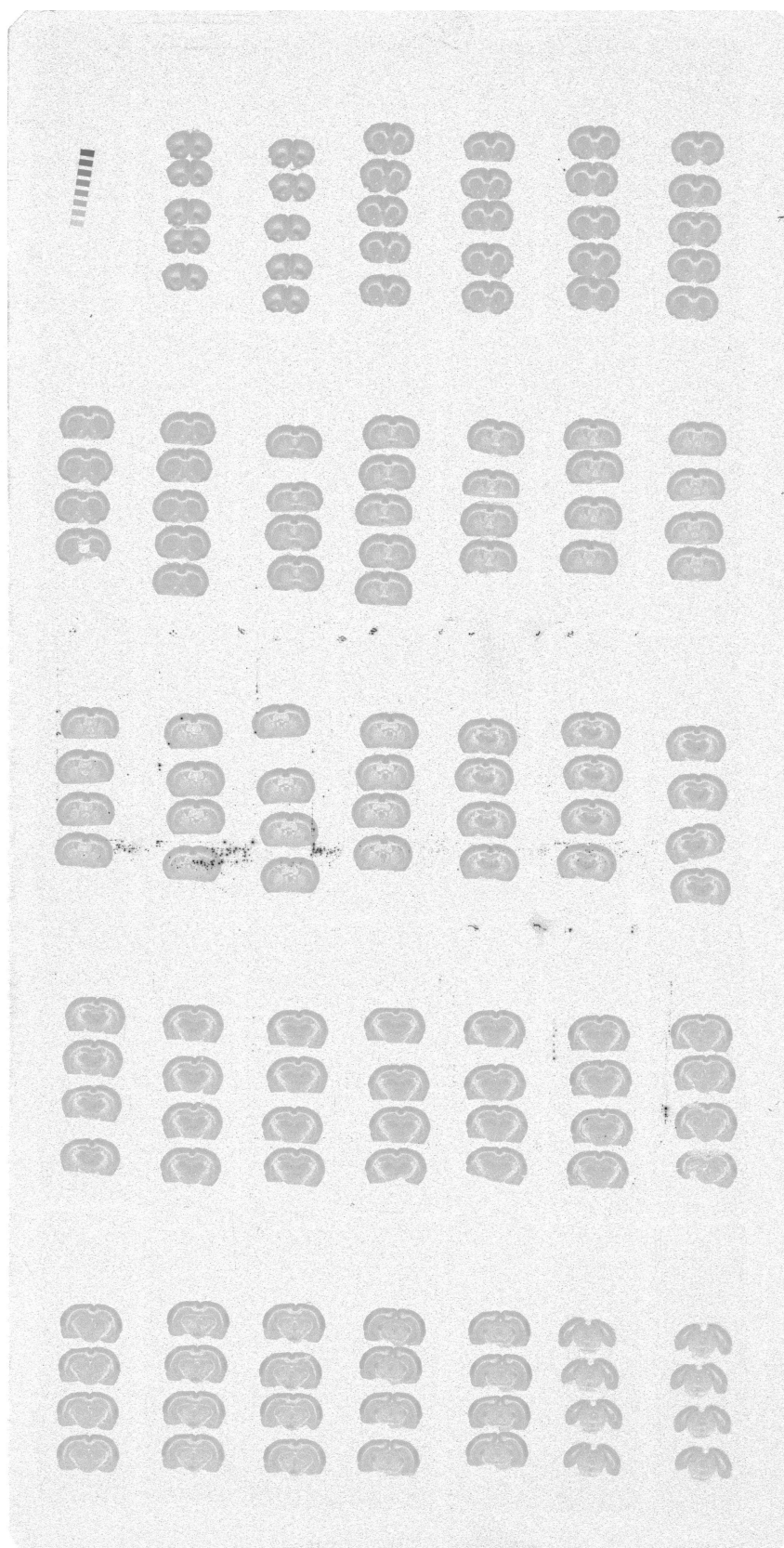
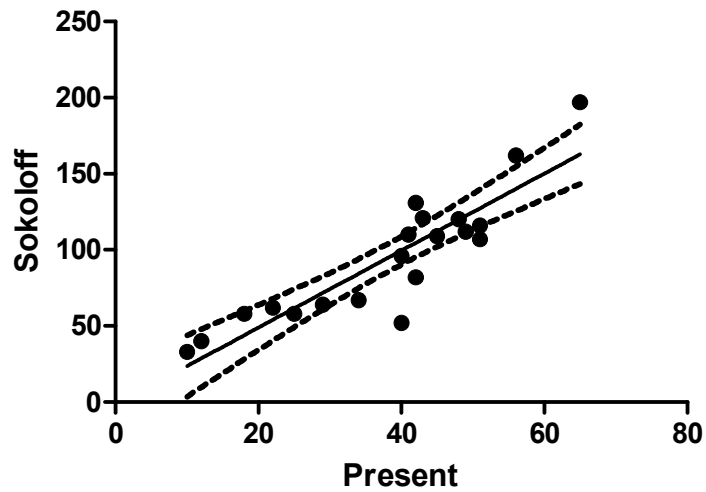


Table 3: Local cerebral glucose utilisation in test subjects (Mean \pm SEM; n=3), compared to reference papers (Sarter, 1990; Sokoloff et al., 1977). Missing SEM values indicates that LCGU in that region could not be identified in all animals.

Region	LCGU $\mu\text{mol}/100\text{g}/\text{min}$	LCGU Sarter	LCGU Sokoloff
Amygdala	40 \pm 4	48 \pm 3	52 \pm 2
Arcuate nucleus	28 \pm 5		
Auditory cortex	56 \pm 5		162 \pm 5
BNST	26 \pm 3		
Hippocampus CA1	36	34 \pm 2	
Hippocampus CA2	37	43 \pm 2	
Hippocampus CA3	36	42 \pm 3	
Caudate putamen	41 \pm 3		110 \pm 4
Central inferior colliculus	71 \pm 4		
Cingulate cortex	54 \pm 7	82 \pm 3	
Corpus callosum	12 \pm 3	19 \pm 2	40 \pm 2
Dentate gyrus	34		67 \pm 3
Dorsal raphe	39 \pm 1	29 \pm 2	
External inferior colliculus	65 \pm 3		197 \pm 10
Infralimbic cortex	51		
Internal capsule	10 \pm 1		33 \pm 2
Lateral geniculate body	40 \pm 7	45 \pm 2	96 \pm 5
Lateral globus pallidus	18 \pm 2	11 \pm 1	58 \pm 2
Lateral hypothalamus	32 \pm 6	33 \pm 3	
Lateral Septum	29 \pm 5	45 \pm 1	64 \pm 3
lateral thalamus	51 \pm 5	52 \pm 3	116 \pm 5
Mammillary body	43 \pm 7	71 \pm 4	121 \pm 5
Medial geniculate body	42 \pm 4	45 \pm 1	131 \pm 5
Median raphe	34 \pm 1		
Motor cortex	48 \pm 3		120 \pm 5
Nucleus Accumbens	42 \pm 4	74 \pm 4	82 \pm 3
Parietal association cortex	49 \pm 6	75 \pm 2	112 \pm 5
Periaqueductal gray	41 \pm 4		
Piriform cortex	54 \pm 4		
Pontine gray matter	22 \pm 1	9 \pm 1	62 \pm 3
Posterior thalamus	51 \pm 6		
Prelimbic cortex	51 \pm 7	119 \pm 3	
PVN	34 \pm 6		
Somatosensory cortex	49 \pm 2		
Substantia nigra	25 \pm 1	12 \pm 1	58 \pm 3
Superior olive	32 \pm 5		
Ventral tegmentum	46		
Ventral thalamus	45 \pm 5	56 \pm 2	109 \pm 5
Ventromedial hypothalamus	33 \pm 4		
Visual cortex	51 \pm 2	49 \pm 4	107 \pm 6

Figure 6: Illustrated correlations between the present data and (a) that of Sokoloff et al. (1977); (b) that of Sarter (1990)

(a)



(b)

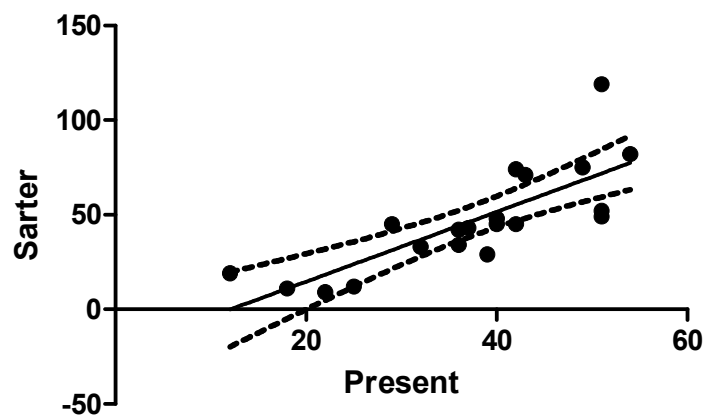


Figure 7: Plasma ^3H ($\mu\text{Ci/ml}$) measured after IV injection of ^3H -2DG in the three rats studied

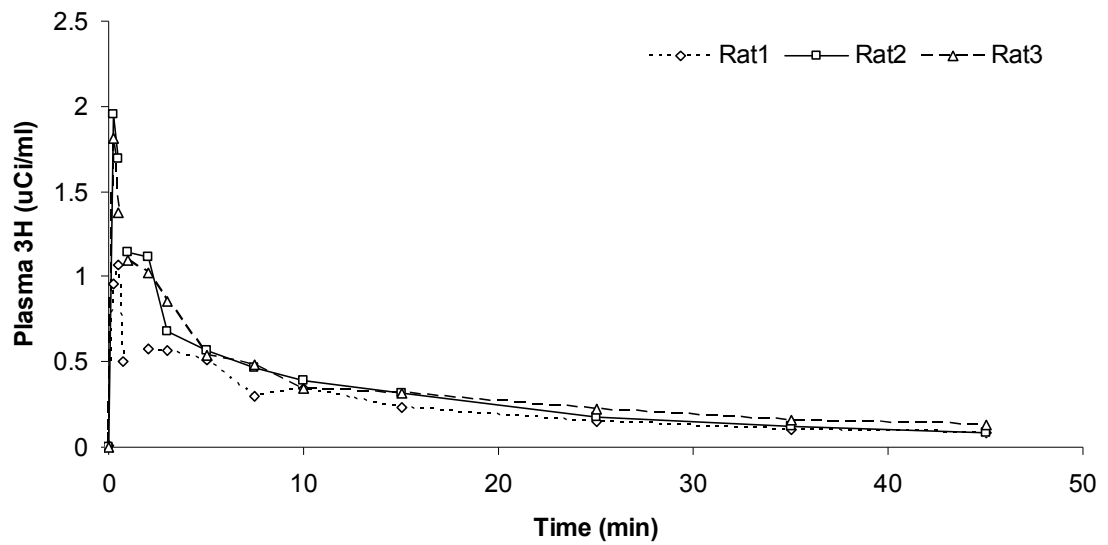
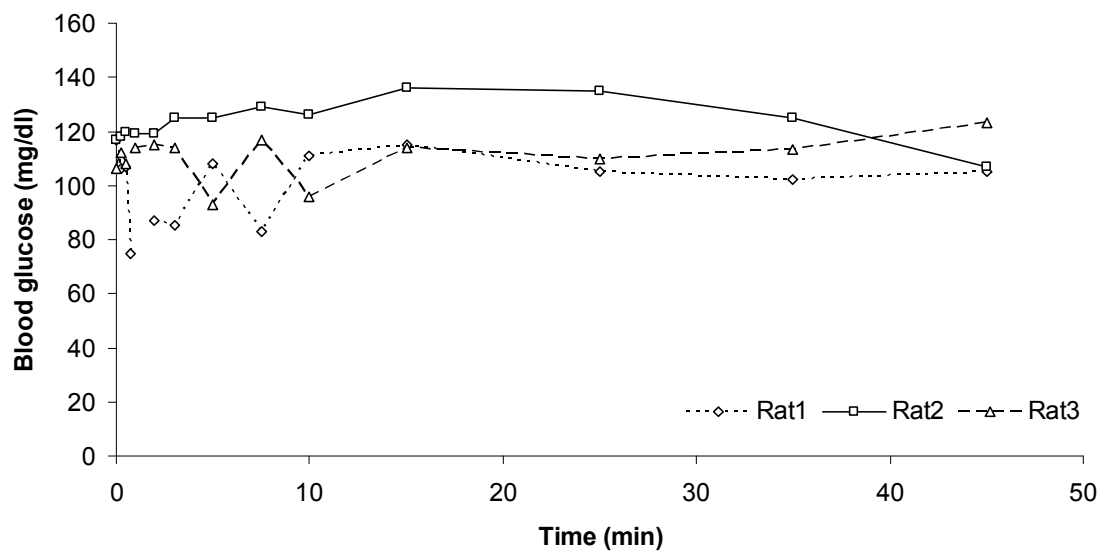


Figure 8: Blood glucose (mg/dl) measured over the course of the LCGU protocol in the three rats studied



Discussion

This study sought to produce data comparable to that in the literature, utilising the safer, cheaper, tritium-labelled analogue of 2DG and replacing film autoradiography through the use of a phosphoimager. The data generated correlated to a high degree with the appropriate literature, and those regions with the lowest and highest LCGU matched those expected, based upon Sokoloff et al.'s (1977) original experiments. A slightly higher level of correlation with the data of Sarter (1990) most likely reflects the use of ^3H -2DG in both studies, with a correspondingly lower specific activity than ^{14}C -2DG as used by Sokoloff et al. (1977).

Although not quantified, for example by measuring plasma corticosterone to examine activation of the HPA axis, during the blood sampling process it appeared that the presence of two investigators and a degree of movement of the swivel was disruptive for the animals. Thus one could expect that these aspects of the present protocol may themselves constitute a stress to the animals. In order to minimise such an effect, it was decided to consider further modifications to the LCGU protocol, reducing the blood sampling load.

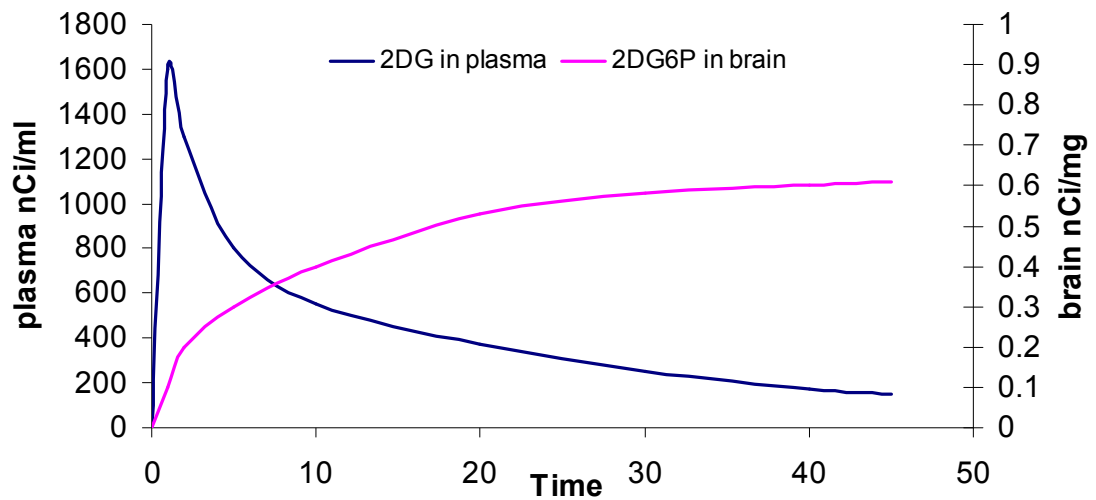
Study 2 - Administration of 2DG by Subcutaneous and Intraperitoneal Routes

Introduction

In order to simplify the LCGU technique, removing the need for surgical implantation of catheters or potentially stressful intravenous (IV) injections (due to the increased intricacy of the procedure the animal must be restrained for longer than by other routes), both subcutaneous (SC) and intraperitoneal (IP) injection of 2DG have been used. The aim of this study was to directly compare the pharmacokinetics of 2DG in mice after either SC or IP administration, in order to assess the suitability of these routes for use. Mice were selected for use in this study in order to reduce the volume of 2DG required, and thus cost. For this reason, mice were used predominantly in the remainder of the work presented in this thesis.

Evidence for the suitability of alternate routes of 2DG administration has been presented in the literature (Meibach et al., 1980), although a direct representation of the pharmacokinetics of 2DG after either SC or IP injection was not included. Comparison of IP injection to IV demonstrated very high correlation between LCGU values via either route, and further demonstrated that a plateau in 2DG uptake was similarly reached regardless of administration route (Meibach et al., 1980). An immediate plasma peak following injection, as with IV (see Figure 9), is not expected as time for absorption of the 2DG from either SC or IP space is required.

Figure 9: Illustration of the expected plasma and brain levels of 2DG and 2DG-6-P after bolus IV injection (adapted from Sokoloff et al., 1977)



Residual unmetabolised 2DG in the plasma or brain after 45 min should also be minimal, assuming that 2DG uptake has reached a plateau. Furthermore, residual 2DG could be expected to be lower after IP injection than after SC (Kelly and McCulloch, 1983b). Residual 2DG in the system could confound measurements of LCGU as it is impossible to dissociate radiolabelled 2DG from its metabolite 2DG-6-P via either autoradiography or scintillation counting. Central levels of radioactivity are expected to rise continuously throughout the 45min period, reaching a plateau as plasma levels of 2DG are exhausted.

Methods

Subjects

Male C57BL/6N mice (Janvier, France) were individually housed in individually-ventilated cages with food and water available ad libitum, under a standard 12h/12h light-dark cycle (lights on 06:00 a.m.), and at a regulated temperature of $22 \pm 0.5^{\circ}\text{C}$ and humidity of $50 \pm 3\%$. The mean bodyweight at testing was $25.6 \pm 0.7\text{g}$. Final testing was carried out between 0700 and 1200 hours. All testing was conducted according to the European Communities Council Directive Nov. 1986 (86/609/EEC) and approved by the animal care and use committee of Johnson & Johnson Pharmaceutical Research and Development.

Measurement of 2DG and 2DG-6-P in Brain and Plasma

2-deoxy-D-[1-³H]glucose (2DG; GE Healthcare, formerly Amersham Biosciences UK, specific activity of 2DG unchanged) (300 µCi/kg) was injected either intraperitoneally or subcutaneously. Following 2DG injection, mice were returned to their home-cage until decapitation.

Mice were decapitated every 5 minutes (n=3 per time point) from 5 to 45 minutes after 2DG injection to obtain time-response curves for the level of 2DG in the plasma and 2DG/2DG-6-P in the brain (towards the end of the 45 minute standard protocol one would expect only negligible amounts of 2DG to remain unmetabolised in the brain, but prior to this measurement of total radioactivity may include both the unmetabolised and metabolised forms).

Upon decapitation, the brain was rapidly removed and stored at –20°C, trunk blood was collected in BD Microtainer K2E tubes (BD Vacutainer Systems, UK), and centrifuged at 1100 g for 10 minutes to separate plasma from red blood cells. Plasma samples were collected for measurement of plasma ³H (residual 2DG) and stored at –80°C.

Brains were homogenized in phosphate-buffered saline (PBS) using glass minibeads and a Mini-BeadBeater-8 (Biospec Products Inc, USA). ³H in plasma and brain samples was measured in duplicate in a TopCount NXT (PerkinElmer, Belgium) microplate scintillation counter after addition of MicroScint-PS scintillant (PerkinElmer, Belgium).

A relative measure of LCGU was calculated as nCi ³H (metabolised 2DG) present per mg of brain tissue.

Results

As expected, a peak in plasma 2DG levels, followed by gradual elimination was seen after injection by either route, corresponding with a time-dependent rise in brain 2DG/2DG-6-P levels reaching a plateau after approximately 25 minutes (Figures 10 and 11).

Also as expected, the peak in plasma 2DG via either injection route occurs substantially later than that expected after intravenous injection (compare with Figure 10). Plasma 2DG peaked within the first 10 minutes via either route, whereas following intravenous injection the peak would be expected within 1 minute.

However, a difference in the plasma 2DG curves between the two routes can be seen in the later time points. Compared to subcutaneous injection, plasma 2DG after intraperitoneal administration appears to reach a lower, more stable level from 35 to 45 minutes.

Figure 10: Measured plasma and brain levels of 2DG and 2DG-6-P after bolus SC injection (data shown: mean lines with individual data, n=3).

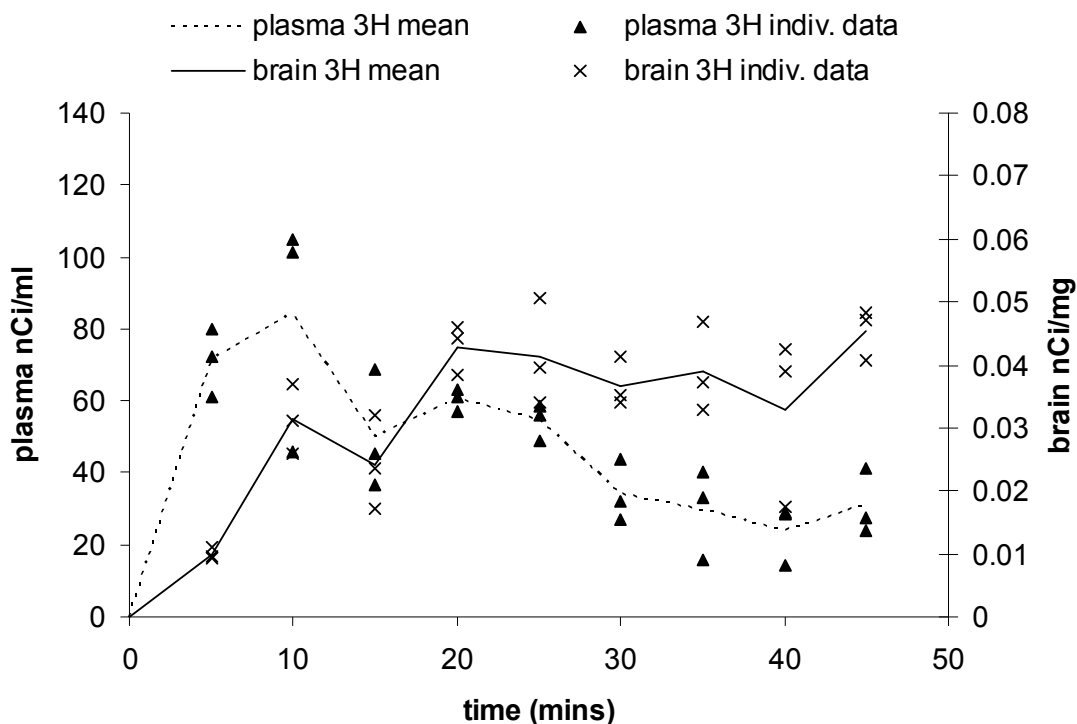
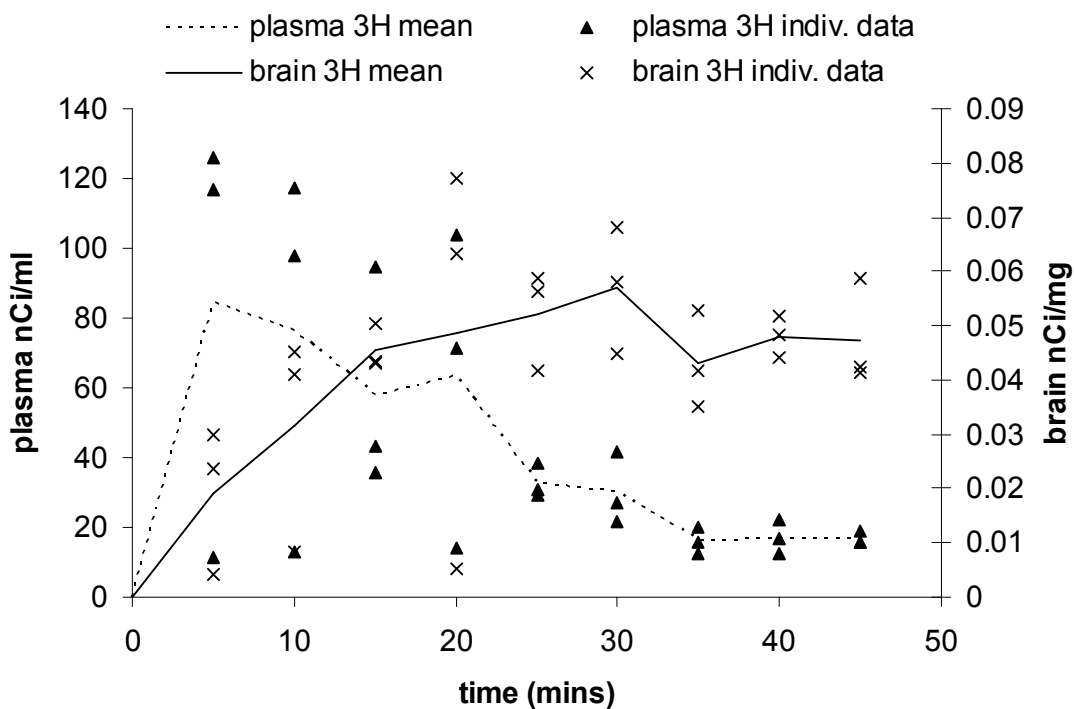


Figure 11: Measured plasma and brain levels of 2DG and 2DG-6-P after bolus IP injection (data shown: mean lines with individual data, n=3).



Discussion

The expected plasma and brain distribution curves were seen following both IP and SC 2DG injection. Measured ^3H levels in the plasma, representing unmetabolised 2DG, peaked early in the 45 minute protocol, and fell with time to minimal levels. Brain ^3H levels, eventually representing 2DG metabolised to 2DG-6-P, plateaued after approximately 25 minutes in both cases, indicating that the duration of the 45 minute protocol is sufficient to reach a steady state, in agreement with Sokoloff et al's (1977) original assumptions. Based on the literature (Kelly and McCulloch, 1983b), it was expected that the IP route would produce lower levels of residual 2DG in the plasma, perhaps due to more complete diffusion and uptake of the ligand via this route. Indeed, in the present study, residual 2DG levels in the plasma speculatively appear lower and more consistent after IP 2DG injection, although this could only be confirmed in a larger-scale study by statistical analysis.

This study has illustrated that the distribution of 2DG and 2DG-6-P in plasma and brain appear to be as expected after either SC or IP administration. Thus, it could be expected that in future studies, a measure of LCGU could be obtained after the injection of 2DG by these routes.

Study 3 - Measurement of LCGU in Dissected Brain Regions by Scintillation Counting

Introduction

As previously discussed, both autoradiography and brain dissection have been used in studies of LCGU, and each has pros and cons.

Autoradiography can be a time-consuming process, requiring sectioning, exposure to film and densitometry to generate data. Using ^{14}C -2DG, imaging requires approximately 5 days, yet does not have the resolution of ^3H -2DG (Gallistel and Nichols, 1983), which requires 3-4 weeks for imaging. The availability of a cryostat for sectioning and a computerised system for densitometry was limited within Johnson & Johnson Pharmaceutical Research & Development, due to ongoing internal projects.

In contrast, the equipment required for dissection of regions of interest from the brain and measurement of LCGU in homogenates was more readily available. In addition to this technical issue, dissection can generate data more rapidly than autoradiography, dependent on the number of samples. However, dissection has a number of clear drawbacks compared to autoradiography. Heterogeneous regions such as the thalamus are grouped into one dissected region, making it more difficult to draw clear conclusions from alterations in LCGU in such regions. Indeed, dissection of these regions may effectively mask changes in LCGU. For example, simultaneous increased LCGU in a particular subregion, with reduced LCGU in another subregion, grouped by dissection, could be missed through averaging to an overall unchanged response. The use of dissection also greatly reduces, and in many cases removes, the ability to measure LCGU in small brain regions. This effect can be twofold, as while a region may be too small to accurately dissect, the amount of radiolabelled 2DG in a small but dissectable region may be too low to accurately count.

In the light of these drawbacks, the aim of the present study was to confirm that LCGU can be reproducibly measured in dissected brain regions, both between different species and by different methods of tissue processing.

For the initial dissection rats were used, due to their larger brain compared to mice, providing a larger amount of brain tissue per dissected region for homogenisation and counting. In addition to confirming that LCGU can successfully be measured in dissected rat brain region, this study compared two methods for the extraction of the radiolabel from the tissue in mice (with the aim of using this species in further studies, reducing cost). The methods compared were mechanical homogenisation and chemical solubilization. Mechanical homogenisation was achieved by rapidly shaking the sample in buffer with glass minibeads. Solubilisation was achieved using a commercially available tissue solubiliser.

Finally, due to the relatively low beta activity of ^3H -2DG for scintillation counting, it was confirmed that increasing the administered dose of 2DG (within Sokoloff et al.'s assumptions) increases the signal to noise/background ratio for counting without further issues.

Methods

Four male Sprague-Dawley rats (Charles River) were used to assess LCGU in dissected brain regions. Housing and treatment was identical to the previous studies. Mean bodyweight at testing was $428 \pm 49\text{g}$. Testing was carried out between 0700 and 1200 hours. The rats received a subcutaneous injection of ^3H -2DG ($300\mu\text{Ci/kg}$) and were returned to their home cage until decapitation 45 minutes later. Following decapitation the brain was rapidly removed and dissected into frontal cortical, septal, hypothalamic, thalamic, hindbrain (region encompassing all tissue posterior to the thalamus excluding the cerebellum - thus this region includes areas of interest including the locus coeruleus, dorsal raphe and other nuclei of relevance to the CRF system), hippocampal, and temporal (to include the amygdala) regions. The brain was sliced longitudinally along the midline, and the frontal cortex dissected, defined as the cortical matter anterior to the corpus callosum. The septum was defined as the tissue between corpus callosum and fornix to the depth of the lateral ventricle. The hypothalamic region was dissected defined posterior to the fornix, anterior to a line between superior colliculus and pituitary, and ventral to the thalamus. The thalamus was also defined posterior to the fornix, anterior to a line

between superior colliculus and pituitary and dissected to the depth of the hippocampus. The hindbrain region was defined as the remaining tissue posterior to the dissected thalamic region. With those regions removed, the hippocampus could be rolled in its entirety from its position inside the cortical fold. Finally the temporal region was defined as a block of tissue taken at the temporal cortex level, to include the amygdala. Illustrative diagrams for these dissections can be found in Appendix 1. The mean standard deviation in the weight of the dissected regions was 6.9 mg.

The dissected regions were weighed (mg wet weight) and stored in 2 ml screw-top plastic vials, initially snap-frozen in liquid nitrogen then stored at -80°C .

The brain regions were thawed and homogenised in phosphate-buffered saline using glass minibeads and a minibead-beater. Following homogenisation, radioactivity levels were counted by liquid scintillation in Microscint 40 scintillation cocktail (Perkin Elmer). LCGU in nCi/mg tissue was calculated.

Male C57BL/6 mice (Janvier) were used to compare the two methods for tissue processing and doses of 2DG. Again, these mice were housed identically to the rats used in the previously described studies. Mean bodyweight at testing was $29.1 \pm 1.6\text{g}$. Testing was carried out between 0700 and 1200 hours.

The regions dissected were the frontal cortex, hypothalamus, thalamus, cerebellum, hippocampus, and the same temporal and hindbrain regions as outlined for the rat brain dissection above. The mean standard deviation in the weight of the dissected regions was 1.9 mg.

To compare tissue processing techniques, dissected regions were either homogenised as previously described for rat brain or solubilised using Solvable (Perkin Elmer) (2DG was injected IP at a dose of $300 \mu\text{Ci/kg}$, and brains removed after 45 minutes). To solubilise the tissue, Solvable was added to the sample vial, and the samples were incubated at 55°C with regular vortexing until the tissue was dissolved. At this point $20 \mu\text{l}$ 30% hydrogen peroxide was added to each sample to reduce colourisation (which could lead to counting errors) and

the samples were incubated for a further 30 minutes. The samples were then transferred to microplates with Microscint 40 scintillant cocktail (Perkin Elmer) and the radioactive content of each sample was then measured in duplicate in a TopCount NXT (Perkin Elmer) microplate scintillation counter.

In their original technique, Sokoloff et al. inject 50 μCi of ^{14}C -2DG to measure LCGU. At the typical range of specific activities for this ligand, this 50 μCi amount equates to approximately 1 μmol of 2DG. This is considered to meet the requirements for tracer theory (Sokoloff et al., 1977), i.e. that molecular concentrations in blood and/or tissues are quantitatively negligible and pharmacologically inactive. At a dose of 300 $\mu\text{Ci/kg}$, a 30 g mouse receives approximately 9 μCi of ^3H -2DG. At typical specific activity, this equates to approximately 0.63 nmol, thus a dose in the range of a thousand fold smaller. Therefore the doubling of the ^3H -2DG dose to 600 μCi , will not constitute a breach of the experimental assumptions, but will benefit the measurement of LCGU by scintillation counting, without excessively increasing the amount of 2DG used.

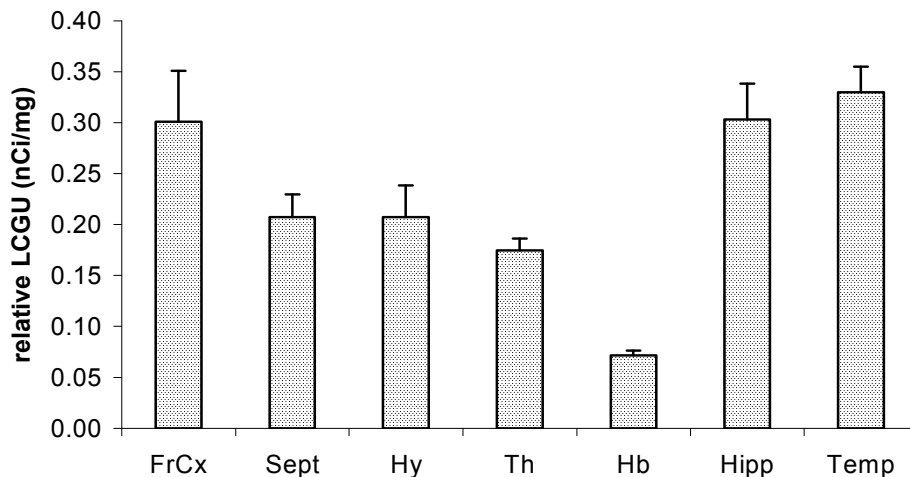
To confirm this experimentally, two mice (data missing for mean bodyweight at testing; estimate 25-30g) were injected with 2DG at either 300 or 600 $\mu\text{Ci/kg}$ IP, brains removed after 45 minutes and LCGU measured using the homogenisation dissection protocol.

Results

A relative measure of LCGU in nCi/mg could readily be measured in dissected rat brain regions, with values ranging from approximately 0.07 to 0.33 nCi/mg (Figure 12) after the subcutaneous dose of 300 $\mu\text{Ci/kg}$ 2DG.

Figure 12: LCGU (nCi/mg) measured in homogenised dissected rat brain regions (n=4)

(legend: FrCx – frontal cortex; Sept – Septum; Hy – hypothalamus; Th – thalamus; Hb – hindbrain region; Hipp – hippocampus; Temp – temporal region)



Both homogenisation (Figure 13) and tissue solubilisation (Figure 14) of dissected mouse brain regions produced values of LCGU similar to those found in rat brain. A similar time-scale is required to process the tissue via either method. According to the manufacturer of Solvable, Perkin Elmer, this tissue processing method should maximise the miscibility of tissue sample and scintillant for efficient scintillation counting (http://las.perkinelmer.com/content/RelatedMaterials/Brochures/BRO_ScintillationCocktailsAndConsumables.pdf). (Chronological note: this individual study was actually conducted after some of the studies investigating the CRF system, having learnt of the availability of Solvable. Therefore, earlier studies into the effects of CRF system manipulation on LCGU use homogenisation, while later studies use Solvable. The confirmed similarity of data produced via either method, and the use of an appropriate control group in each study allows the comparison of the data.)

Doubling of the dose of 2DG injected from 300 to 600 $\mu\text{Ci/kg}$ increased the relative level of LCGU measured by approximately two-fold (Figure 15).

Figure 13: LCGU (nCi/mg) measured in homogenised dissected mouse brain regions (n=8)

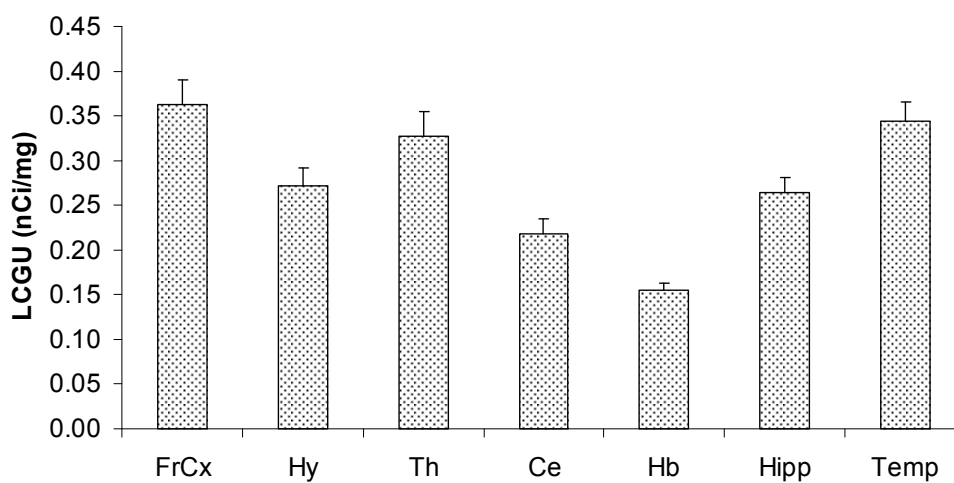


Figure 14: LCGU (nCi/mg) measured in solubilised dissected mouse brain regions (n=3)

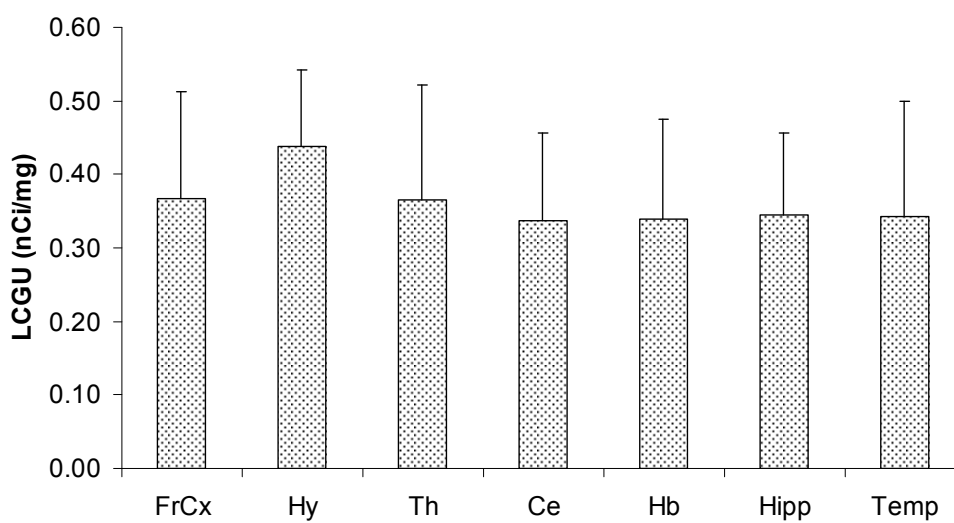
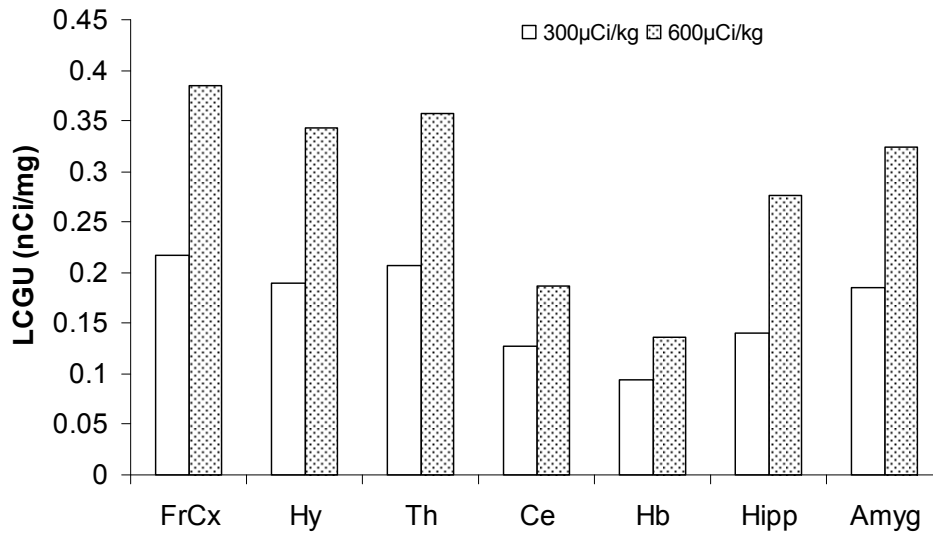


Figure 15: LCGU (nCi/mg) measured in homogenised dissected mouse brain regions (n=1/dose)



Discussion

This study demonstrated that a measure of LCGU can be reproducibly obtained from dissected brain regions in either rat or mouse after peripheral administration of 2DG. This is in agreement with previously reported studies, where a high correlation between LCGU values obtained through either autoradiography or dissection was found (Meibach et al., 1980). The suitability of a region for dissection is determined largely by size, of which a consequence is containing sufficient metabolised 2DG to be detectable by scintillation counting.

The levels of LCGU in the dissected regions in relation to one another resemble those reported by Sokoloff et al (1977), when the appropriate individual regions are grouped as dissected in the present study. The frontal cortex and thalamus are regions with higher metabolism than the hypothalamus and hippocampus (Sokoloff et al., 1977). While LCGU in the amygdala is reported by Sokoloff et al. (1977) to be approximately half that of the frontal cortex, the temporal region dissected presently to include the amygdala also contains cortical matter, which typically has a higher LCGU. Thus the mean LCGU for the dissected temporal region is higher than that expected for the amygdala alone. This highlights a principal drawback of the dissection technique, the loss of neuroanatomical resolution. This problem is particularly apparent in the

dissected hindbrain region, which amalgamates numerous small brain regions and nuclei, many of which are reported to have high LCGU. However, the apparent increased hindbrain LCGU found using tissue solubilisation highlights a further issue – the effectiveness of dissection and homogenisation techniques in measuring the level of 2DG uptake. As a region with an increased mass compared to the other dissected regions, the solubilisation method may be more effective in recovering 2DG from within the cells.

The reduction in neuroanatomical resolution introduced by dissecting brain regions makes it impossible to examine the involvement of small regions, either non-dissectable or grouped into larger dissected regions, in changes in LCGU. As such small regions may contain large amounts of neurons expressing particular neurotransmitters, the opportunity to implicate these particular neurotransmitters in LCGU changes is also lost. Dissection does, however, remove the need for cryostat and densitometry hardware, which were less readily available than homogenisation and scintillation counting equipment.

In order to avoid accessibility issues with the equipment for autoradiography, the dissection version of the original LCGU technique will be further used to study the effect of manipulating the CRF system on LCGU. However, due to the noted drawbacks of this technique, crucial results will be confirmed by autoradiography.

Study 4 - The Effect of 2DG on the HPA Axis

Introduction

Through competition with intracellular glucose, 2DG inhibits phosphohexose isomerase (Sols and Crane, 1954; Tower, 1958), the enzyme that converts phosphoglucose to phosphofructose, and thereby blocks glycolysis at the initiation stage. Therefore, at sufficient doses, 2DG is expected to cause depletion of ATP as well as of glucose derivatives required for protein glycosylation (Kang and Hwang, 2006). This energy depletion constitutes a pharmacological stressor, and it should be noted that in sufficiently high doses, 2DG itself activates the HPA axis (Weidenfeld et al., 1984; Weidenfeld et al., 1994; Khan and Watts, 2004). However, the dose required for this effect is several hundred-fold greater than the dose used to measure LCGU. 2DG was capable of activating the HPA axis at doses >100 mg/kg, while the tracer amount of 2DG used to measure LCGU equates to approximately 6 µg/kg (at the 600 µCi/kg dose). The aim of this study was to confirm that the doses of 2DG used to measure LCGU do not affect the HPA axis.

Methods

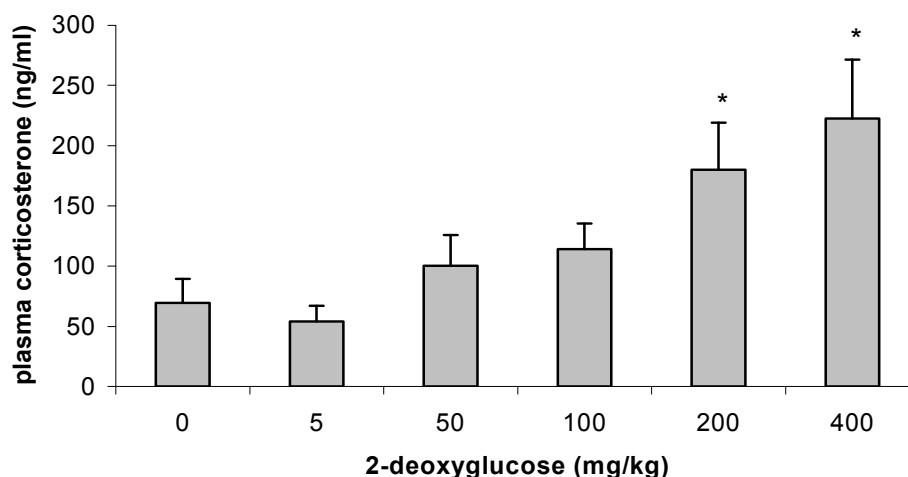
To confirm that low doses of 2DG do not affect the HPA axis, 2-Deoxy-D-glucose (non-radiolabelled; Sigma Aldrich, Germany) was injected intraperitoneally in C57BL/6N mice (Janvier; housed identically to those used in the previous studies; mean body weight at testing 27.1 ± 0.97 g) at doses from 5-400 mg/kg in saline vehicle. The lowest dose of 5 mg/kg tested is close to one thousand times greater than the dose used to measure LCGU, and the higher end of the dose range was chosen to include doses expected to increase plasma corticosterone, based on studies in the literature (Weidenfeld et al., 1984; Weidenfeld et al., 1994; Khan and Watts, 2004). After 45 minutes plasma was collected as previously described for the measurement of plasma corticosterone. All testing was carried out between 0800 and 1200 hours. Plasma corticosterone was measured using ImmuchemTM Double Antibody corticosterone ¹²⁵I RIA kits (MP Biomedicals, USA). Data were statistically analysed non-parametrically using the Kruskal-Wallis (KW) test (SPSS v13.0, SPSS Belux, Belgium). Where

appropriate, post-hoc comparison was performed by the Mann-Whitney test (2-sided).

Results

Intraperitoneal administration of unlabelled 2DG resulted in a dose-response increase in plasma corticosterone after 45 minutes (Figure 16), only reaching statistical significance at the 200 and 400 mg/kg doses ($p=0.026$ and 0.015) (equivalent to approximately 20 Ci/kg and 40 Ci/kg, respectively, when compared to 2DG used to measure LCGU at a typical specific activity of 8 Ci/mmol), more than one thousand-fold higher than the dose used to measure LCGU (approx 6 μ g/kg at an injected dose of 600 μ Ci/kg).

Figure 16: The effect of intraperitoneally administered 2DG on the HPA axis. (n=6/group). * $p<0.05$.



Discussion

The results of this study clearly demonstrate that 2DG only affects the HPA axis, illustrated by increased plasma corticosterone, at substantially higher doses than those used to measure LCGU. Thus, effects on the HPA axis in subsequent studies cannot be attributed to the use of 2DG to measure LCGU. While the present study does not measure other centrally mediated parameters that could be altered by 2DG itself, such as LCGU, the experimental design of such studies should ensure that any such effects are of negligible consequence. The dose of 2DG used to measure LCGU is considered to meet the requirements for tracer theory, defined as the presence of the compound at

levels below those of pharmacological significance, and furthermore, all treatment groups, including control, in subsequent studies will receive an identical dose of 2DG.

The effects of 2DG on the HPA axis in the present study agree with previously reported data (Weidenfeld et al., 1984; Weidenfeld et al., 1994; Khan and Watts, 2004), in which 2DG was capable of activating the HPA axis at doses >100 mg/kg. Through competition with intracellular glucose, 2DG inhibits phosphohexose isomerase (Sols and Crane, 1954; Tower, 1958), the enzyme that converts phosphoglucose to phosphofructose, and thereby blocks glycolysis at the initiation stage. Therefore, at sufficient doses, 2DG is expected to cause depletion of ATP as well as of glucose derivatives required for protein glycosylation (Kang and Hwang, 2006), constituting a pharmacological stressor through energy depletion. The use of a tracer dose of 2DG which is only sufficient for the measurement of LCGU ensures that this effect is avoided in studies of central glucose metabolism.

Study 5 - Assessment of a Method for Free-hand ICV Injection in mice

Introduction

Due to the inability of peripherally injected peptides to cross the blood-brain barrier, the potential for these peptides to be broken down in the periphery and the very limited availability of small molecule agonists and antagonists for CRF₁ and CRF₂ receptors that would negate the problems of stability and the blood-brain barrier, it is necessary to administer the CRF peptides and derived antagonists directly into the brain. This is most commonly achieved by injecting these peptides into the lateral ventricle (intracerebroventricular, or ICV injection) through a surgically implanted cannula. This method allows single and repeated ICV injections usually through the use of a separate injection cannula and a dummy cannula to occlude the guide cannula between uses. Drawbacks of this method include: the necessity for surgery and a suitable recovery period, the risk of cannula blockage or loss before or during studies, and a degree of stress on the animal during the process of injecting compounds through the cannula. Experience of surgically implanted cannulas for ICV injection within Johnson & Johnson Pharmaceutical Research & Development suggested that the blockage of cannulas in mice between surgery and testing was a repetitive problem, and that activation of the HPA axis by the handling during injection was often sufficient to mask treatment-induced effects. However, a method for the ICV administration of compounds without the need for surgery has been reported in the literature (Pelleymounter et al., 2000; Pelleymounter et al., 2004). Using this method the need to surgically implant cannulas is avoided, although the obvious drawbacks include the need to anaesthetise the animal while inserting a needle directly through skin and skull into the lateral ventricle, aspects which could themselves influence LCGU, and activate the HPA axis. Therefore, the aim of this study was to assess the suitability of this method for use in combination with measuring LCGU.

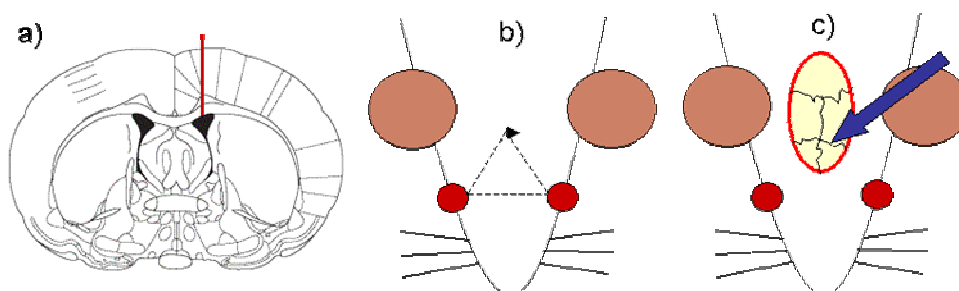
Methods

Male C57BL/6N mice (Janvier, France) were used under the same conditions as for previous studies. Mean body weight at testing 26.9 ± 1.0 g. All testing was carried out between 0800 and 1200 hours.

The freehand ICV procedure was adapted from Pellemounter et al (Pellemounter et al., 2000; Pellemounter et al., 2004), who used a Hamilton 10 μ l syringe with a 30 gauge needle (limited to 4mm using polyethylene tubing) to inject into the third cerebral ventricle directly under isoflurane anaesthesia. Pilot studies suggested that this method of injection risked penetrating the cerebral vein running along the midline, so the technique was adapted for injecting into the lateral ventricle.

In the modified technique, the mice were weighed, then briefly (30 seconds or until a clear change in breathing rhythm was seen) anaesthetized using isoflurane (4% in 30% O₂, 70% N₂O) until the change of breathing rhythm characteristic of full anaesthesia was apparent. 5 μ l of solution was injected using a Hamilton 10 μ l syringe and 30-gauge needle limited to 2.5 mm length using a piece of polyethylene tubing. Using downwards pressure above the ears the head was stabilised, and the needle was inserted directly through the skin and skull into the lateral ventricle (see Figure 17a), targeted by visualizing an equilateral triangle between the eyes and center of skull to locate bregma, then inserting the needle 1.5-2 mm laterally to this point (illustrated in Figure 17b). This ensures penetration of the skull at the suture line of the skull (Figure 17c) plates to minimize necessary force, while avoiding the centrally located vein. 5 μ l was injected over an approximately 5 sec period, followed by a 10 sec delay to allow diffusion and prevent backflow.

Figure 17: Illustrations of the freehand ICV injection technique: a) Paxinos & Watson brain atlas slide (at bregma) illustrating the penetration of the needle into the lateral ventricle (black space); b) location of the injection site; c) injection site relative to skull sutures



In a pilot study injecting methylene blue dye into the lateral ventricles of thirty C57BL/6N mice (Janvier, mean body weight $26.6 \pm 1.1\text{g}$) using this method, the success rate (as determined by the presence of blue dye in the lateral ventricles after removal and slicing of the brain) of injection was 93.3%, which corresponds with the values reported in the literature (approximately 95%; Pellemounter et al., 2000). Under normal experimental circumstances an animal with any evidence of subdural haemorrhage (altered physical activity, blood upon dissection) would be excluded from analysis (as in Pellemounter et al., 2000; Pellemounter et al., 2004), however in this pilot study no such evidence was seen while targeting the lateral ventricle.

In order to assess the effectiveness of the ICV injection technique for compound delivery, the effect of CRF and amphetamine on behaviour in a novel open field was measured.

The open field apparatus consists of four transparent plastic arenas (26 x 26 x 38 cm) surrounded by infrared beam grids to allow the measurement of horizontal and vertical activity. Activity is recorded using Tru-Scan Software Version 1.011 (Coulbourn instruments, Allentown, USA). Each arena is separated from the others and from the experimenter with opaque panels. Parameters of locomotor activity and rearing are recorded over 30 minutes in 6 minute time bins.

To examine the effects of the components of the injection procedure, mice received either no treatment, anaesthesia only, anaesthesia plus needle insertion only or a full ICV injection of 5 μl saline. To assess the effectiveness of the method to deliver an active dose of compound, separate groups of mice received either ICV injections of 0.5 μg CRF (in 5 μl saline), or 20 μg amphetamine (in 5 μl saline). After treatment, mice were returned to their home cage for 10 minutes to recover before being placed in the open field apparatus for 30 minutes.

To assess the effects of components of the injection procedure or pharmacological treatment on the level of HPA axis activation after exposure to

the open field, plasma corticosterone was measured after exposure to the open field using Immuchem™ Double Antibody corticosterone ¹²⁵I RIA kits (MP Biomedicals, USA).

To assess whether the injection technique produced significant effects on LCGU measurements, two groups of mice received either an ICV injection of saline using the free-hand method described above, or no injection (i.e. no component of the ICV procedure), and an IP injection of 300 µCi/kg ³H-2DG. 45 minutes later the brain was removed and dissected for measurement of LCGU using the protocol described above. Blood glucose after the 45 minute protocol was measured using a commercially available glucose meter (Lifescan), and residual 2DG content in the plasma was measured by scintillation counting. The effect of the brief anaesthesia alone, or that of needle insertion, on LCGU was not measured in this study as it is the effect of the entire ICV injection procedure that must be accounted for in future studies (i.e. in order to later examine the effect of ICV CRF compared to ICV saline injection, anaesthesia or needle insertion alone will not be of use in delivering the compound). It has been established in the literature that prolonged anaesthesia throughout the course of measuring LCGU (i.e. at least 45 min) significantly decreases LCGU globally throughout the brain (Frietsch et al., 2000).

Overall effects on LCGU, residual 2DG, blood glucose, total distance traveled and total rearing behaviour in the open field, and on plasma corticosterone were compared using the Kruskal-Wallis test, and Mann-Whitney test for post-hoc comparisons, with a significance level of $p=0.05$ in SPSS. Effects on distance traveled and rearing in the open field by 6 minute time bins were analysed using repeated measures ANOVA with Greenhouse-Geisser correction for sphericity where appropriate (Mauchly's W test $p<0.05$), with t-tests for post-hoc comparisons in SPSS with a significance level of $p=0.05$.

Results

In the open field, total distance moved was slightly reduced in animals that received a component of the ICV injection, compared to untreated animals ($p<0.009$) (Figure 18). However, individual time bin analysis revealed that this

reduction was predominantly due to differences during the first 12 minutes in the open field (Figure 20; repeated measures ANOVA time effect $F_{4,88}=5.794$, $\epsilon=0.655$; $p=0.002$, treatment effect $F_{3,22}=12.183$; $p<0.001$, treatment-time interaction effect $F_{4,88}=9.562$, $\epsilon=0.655$; $p<0.001$), although reduced locomotion continued until the 18 minute time bin ($p<0.039$). Similar effects were seen on overall rearing behaviour, with reduced levels in animals receiving anaesthesia plus needle or full ICV injection ($p=0.002$) (Figure 19), also due to effects in the first 12 minutes (Figure 21; repeated measures ANOVA time effect $F_{4,88}=10.543$; $p<0.001$, treatment effect $F_{3,22}=10.291$; $p<0.001$, treatment-time interaction effect $F_{4,88}=7.030$; $p=0.002$). ICV treatment with 0.5 μg CRF greatly reduced both total distance moved and rearing compared to both saline ICV treated and untreated animals ($p=0.001$ vs. saline ICV). In contrast, amphetamine induced a trend for increased distance moved ($p=0.073$) while reducing rearing ($p=0.001$). These effects were present across all time bins (Figures 22 and 23; distance repeated measures ANOVA time effect $F_{4,72}=6.502$, $\epsilon=0.535$; $p=0.003$, treatment effect $F_{2,18}=12.167$; $p<0.001$; rearing repeated measures ANOVA time effect $F_{4,72}=5.995$, $\epsilon=0.733$; $p=0.001$, treatment effect $F_{2,18}=30.381$; $p<0.001$, treatment-time interaction effect $F_{4,72}=3.156$, $\epsilon=0.733$; $p=0.011$).

Plasma corticosterone after exposure to the open field was unaffected by any of the components of the ICV procedure (Figure 24), but was significantly increased by either CRF or amphetamine treatment ($p<0.005$). Plasma corticosterone levels in saline-injected mice were 228 ± 23 ng/ml (mean \pm SEM), while those in CRF- and amphetamine-treated mice were 316 ± 11 and 316 ± 6 ng/ml, respectively.

The ICV injection procedure had no significant effect on LCGU in any of the regions dissected (Figure 25), and no effect on blood glucose (untreated mean 125 ± 8 mg/dl, ICV 120 ± 5 mg/dl) or residual plasma 2DG (untreated mean 79 ± 21 nCi/ml, ICV 69 ± 5 nCi/ml).

Figure 18: The effect of components of the ICV injection procedure, and CRF or amphetamine treatment on total distance travelled in the open field apparatus (n=6-7/group)

(None – untreated; Anaesth – anaesthesia only; Needle – anaesthesia plus needle insertion) (#: $p < 0.05$ vs. None; *: $p < 0.05$ vs. Saline)

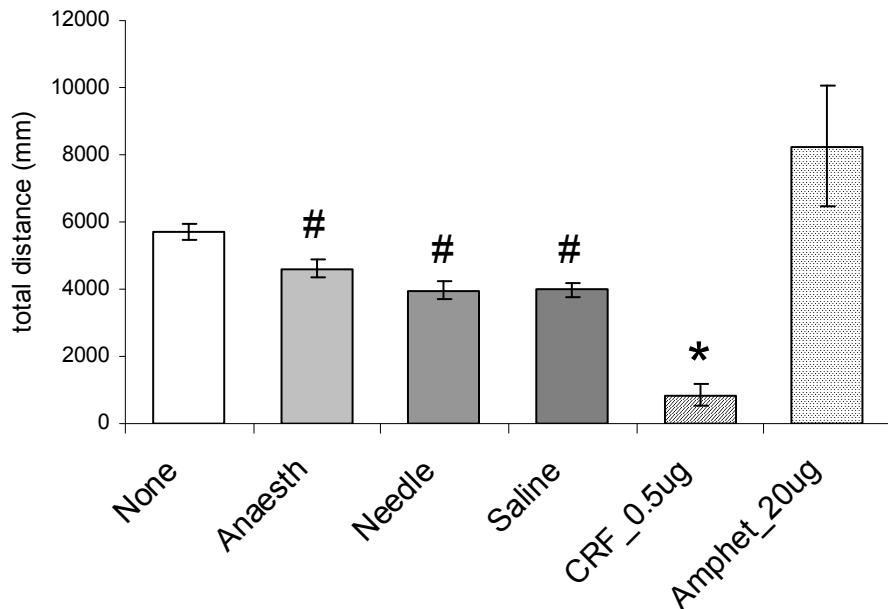


Figure 19: The effect of components of the ICV injection procedure, and CRF or amphetamine treatment on overall rearing behaviour in the open field apparatus (n=6-7/group) (#: $p < 0.05$ vs. None; *: $p < 0.05$ vs. Saline)

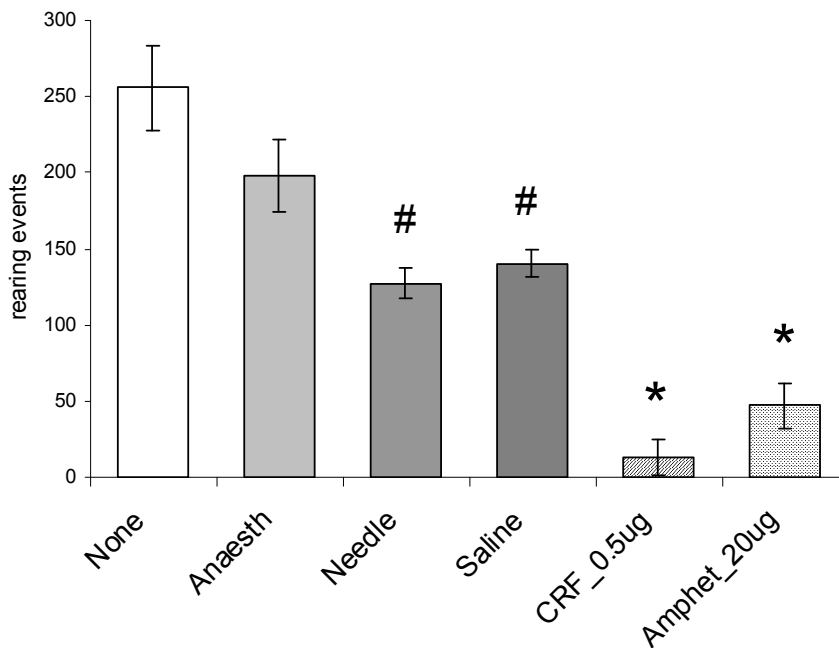


Figure 20: The effect of components of the ICV injection procedure on distance travelled in the open field apparatus (n=6-7/group) (post-hoc effects - #: treatment effect vs. None; *: within-group time effect)

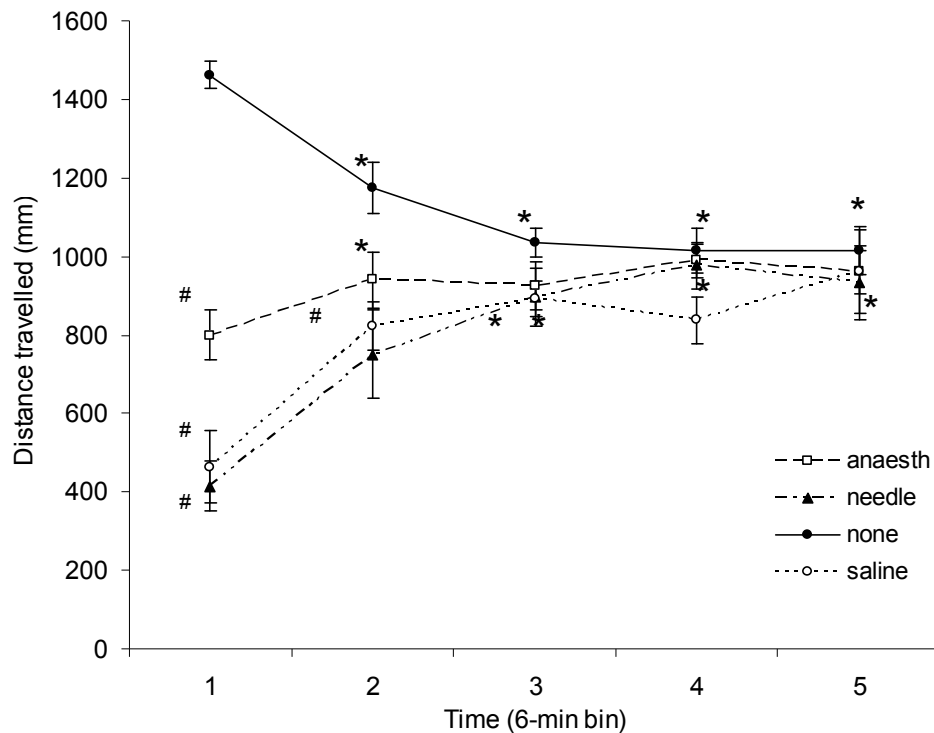


Figure 21: The effect of components of the ICV injection procedure on rearing behaviour in the open field apparatus (n=6-7/group) post-hoc effects - #: treatment effect vs. None; *: within-group time effect)

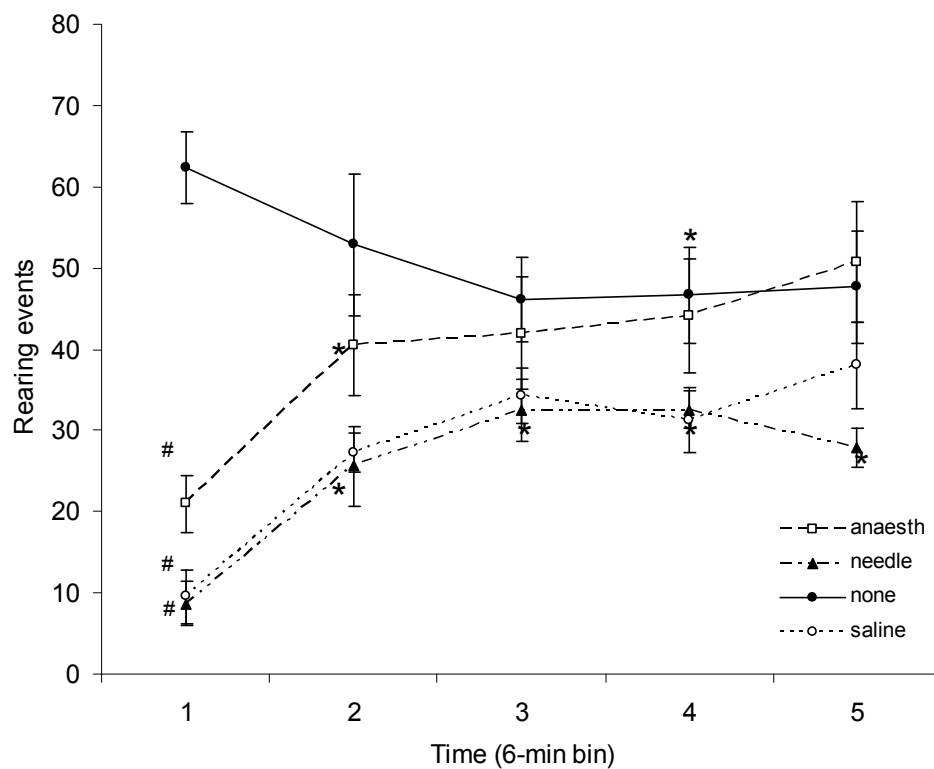


Figure 22: The effect of CRF or amphetamine treatment on distance travelled in the open field apparatus (n=6-7/group) (post-hoc effects - #: treatment effect vs. None; *: within-group time effect)

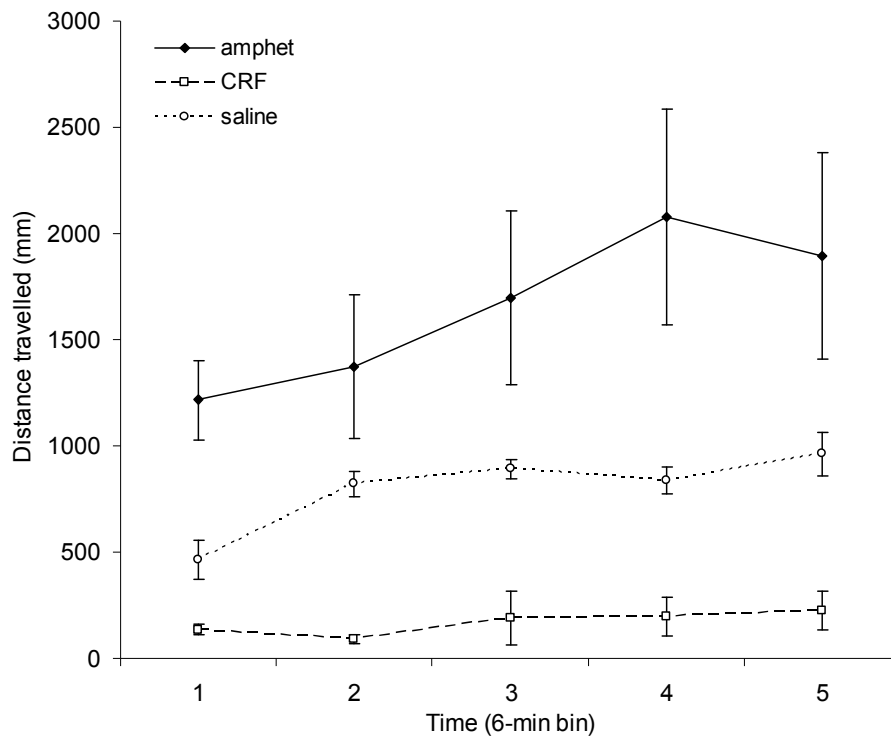


Figure 23: The effect of CRF or amphetamine treatment on rearing behaviour in the open field apparatus (n=6-7/group) (post-hoc effects - #: treatment effect vs. None; *: within-group time effect)

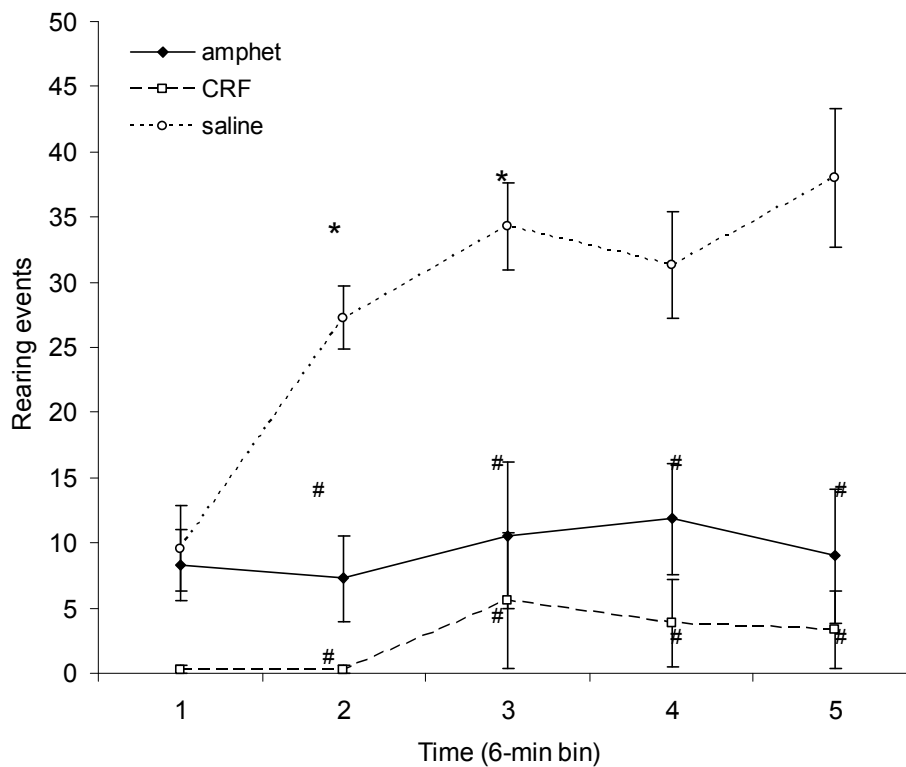


Figure 24: The effect of components of the ICV injection procedure, and CRF or amphetamine treatment on plasma corticosterone levels after exposure to the open field apparatus (n=6-7/group) (**: $p < 0.005$ vs. Saline)

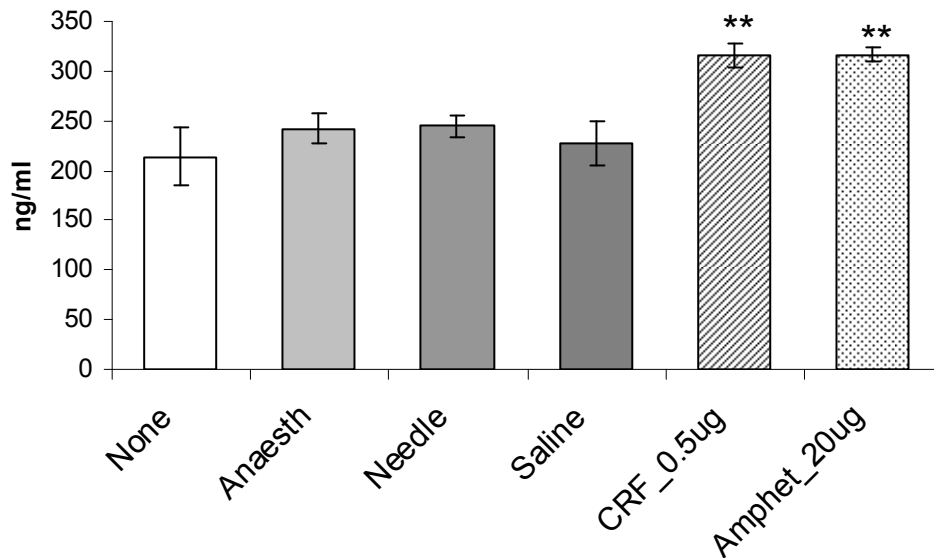
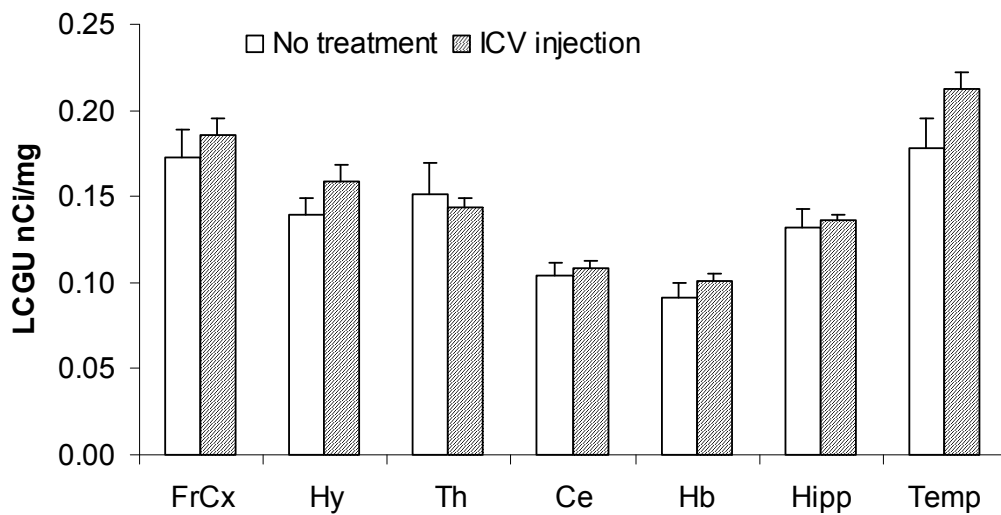


Figure 25: The effect of the ICV injection procedure on LCGU in dissected mouse brain regions (n=11 no treatment, n=16 ICV)

(legend: FrCx – frontal cortex; Hy – hypothalamus; Th – thalamus; Ce – Cerebellum; Hb – hindbrain region; Hipp – hippocampus; Temp – temporal region)



Discussion

This study assessed the effectiveness of a freehand method for ICV injection, requiring no surgical intervention. Using this technique it was possible to reliably deliver compound into the lateral ventricle of mice, and it was possible to detect a pharmacological effect of CRF administration. However, there are clear drawbacks of this technique, which were further illustrated in the open field paradigm.

The freehand ICV injection procedure used in the present study can be broken down into three component parts: anaesthesia, the penetration of skin and skull with a needle, and the injection of saline or another compound. As was seen in the open field paradigm, these components either alone or together, produced significant effects on behaviour, without the addition of a compound with a pharmacological mechanism of action. All of these components reduced overall locomotor activity in the open field, predominantly in the first 12 minutes after injection, with anaesthesia alone affecting locomotion to a slightly lesser degree than the other two components. Anaesthesia alone did not significantly affect overall rearing, although there were clear effects in the first 12 minutes as for locomotion. However, both needle insertion and further saline injection clearly reduced rearing to a greater degree. These effects are comparable to those of a stressor on behaviour in the open field (Carli et al., 1989; Broqua et al., 1992), which may serve to limit the freehand ICV technique's usefulness in studying stress-related behaviour or components of the stress system. The lack of a group with implanted cannulae for comparison is a flaw in the present study. The inclusion of such a group in a further study would allow the direct comparison of a full ICV injection between the two methodologies.

While the effects of the ICV injection components diminish with time in the open field, it may not be suitable to allow a longer recovery period between injection and testing, as it is typically desirable to examine the effects of a compound delivered via the ICV route soon after injection (e.g. after 10 minutes recovery as used here). Indeed, when using a paradigm such as the measurement of LCGU, it is desirable to begin the protocol immediately after treatment.

Despite the effects of the ICV procedure itself on behaviour in the open field, the effects of CRF treatment were visible throughout the exposure to the open field, with further reductions in locomotor activity and rearing, suggesting an anxiogenic-like effect. Indeed, CRF has previously been reported to reduce locomotor activity in a novel open field environment (Dunn and Berridge, 1990), matching the present data. CRF also further activated the HPA axis, above the level induced by exposure to the open field or the components of the ICV procedure, as indicated by increased plasma corticosterone levels, and also in agreement with the literature (Dunn and Berridge, 1990). At this point a limitation of the present study should be noted, namely that the effects of the ICV procedure and those of CRF and amphetamine on the HPA axis were only measured after the open field paradigm. As exposure to the open field itself activates the HPA axis (as demonstrated by plasma corticosterone levels in the unmanipulated control group, which at approximately 200 ng/ml far exceed those levels expected at true baseline, of close to zero) the effects of the ICV procedure on this measure are likely to be masked.

Based on the literature, amphetamine is expected to produce a non-specific increase in locomotor activity (Swerdlow et al., 1993). As there was no significant effect of amphetamine treatment on locomotor behaviour in the present study, only a trend, it is not possible to conclude that a clear pharmacological effect was produced, in contrast to the effects of CRF treatment. This may indicate that the effects of the ICV procedure on behaviour in the open field conflict with those expected after amphetamine administration, and could suggest that the presently used ICV injection is not ideal for the study of such pharmacological manipulations. However, the failure of amphetamine to elicit a significant effect on locomotion in the present study may also represent a sub-effective dose, insufficient experimental power or excessive variability through insufficient group sizes. The contribution of these issues to the measured effect of amphetamine could be investigated in further studies.

A similar freehand method for ICV injection has been used to study the effects of CRF on anorectic behaviour and the effects of Urocortin 2 and 3 on anxiety-related behaviour and the HPA axis (Pelleymounter et al., 2000;

Pelleymounter et al., 2004). In these studies, the effects of the injected peptides were significant when compared to mice receiving ICV injections of vehicle, after similar recovery times (15-30 minutes) to those used in the present study. This supports the usefulness of this technique in studying the effects of CRF and the Urocortins after ICV administration.

In the present study, the ICV injection procedure did not detectably influence LCGU. Stress has been reported to have clear effects on LCGU in the literature (Soncrant et al., 1988; Xing et al., 1990; Duncan et al., 1993; Frietsch et al., 2000; Takamatsu et al., 2003a, 2003b; and considered later in this thesis). Therefore, the lack of a clearly detectable effect of the ICV procedure on LCGU suggests that the degree of stress induced by the procedure is not sufficient to significantly alter LCGU. While the effects of components of the ICV procedure on LCGU were not individually studied, as the technique in its entirety is required for studies into the effects of ICV injected peptides, the effect of anaesthesia on LCGU has been considered in the literature. LCGU is greatly reduced in a global manner when measured under anaesthesia (Sokoloff et al., 1977; Frietsch et al., 2000). However, the effects of a brief anaesthesia on LCGU have not been studied alone, although in studies utilising Sokoloff et al.'s (1977) original method, rats typically have intravenous and arterial catheters implanted under anaesthesia hours before the measurement of LCGU, while maintaining significant LCGU responses to pharmacological treatment.

Blood glucose was measured after the LCGU protocol as constant blood glucose was a requirement of Sokoloff et al.'s original technique (Sokoloff et al., 1977). The consequence of large alterations in the level of blood glucose, brought about through either physical or pharmacological means, could be variation in the level of competition with injected 2DG, leading to incorrect estimates of LCGU. Blood glucose at the end of the LCGU protocol was unaltered by ICV injection, suggesting that the experimental assumption was met. The measurement of residual 2DG in the plasma after the LCGU protocol provides a further indication as to whether abnormal competition with glucose has occurred, as (for example) increased blood glucose competing with 2DG may lead to higher amounts of residual, unmetabolised, 2DG. However, as the 45 minute protocol is designed to allow ample time for injected 2DG to be

metabolised (indicated by the plateau in metabolised 2DG-6-P levels in the brain, see Figures 10 and 11), only large variations in blood glucose may be sufficient to influence this variable.

Via the direct penetration of skin and skull, the freehand ICV technique could introduce pathogens into the brain, potentially leading to an immune response, itself with consequences on stress levels or LCGU. However, the exclusive use of this freehand ICV technique in acute studies lasting up to one hour before sacrifice should serve to minimise the size of such an effect. Furthermore, the use of surgically implanted cannulae cannot definitively prevent this problem, as the presence of a direct channel into the brain over a period of (up to) weeks also provides a chance for the introduction of pathogens. As long as the guide cannula is sealed with a dummy cannula the risk is minimised, however removal of the dummy cannula to prevent its mechanical seizure within the guide, or in the case of damage, or indeed to insert the injection cannula for testing, provides a chance for the introduction of pathogens. The introduction of pathogens into the animal's body is a risk during any injection, for example also via subcutaneous or intraperitoneal injection, yet this factor is not typically considered a serious issue, at least in acute studies.

In conclusion, the evidence from this study suggests that the freehand ICV injection procedure adapted from Pelley et al. (2000; 2004) is an alternative to surgically implanted cannulas for the central administration of compounds in mice. This freehand method has clear disadvantages, including itself affecting behaviour in a stress-related paradigm, the open field, and disrupting the expected effects of amphetamine in the present studies. However, as no significant effects of an ICV injection using this technique were seen in LCGU, and pharmacological effects of CRF administration were detectable, it was decided to further use this technique despite its disadvantages, to negate the need for surgery.

Summary & Conclusions

In this series of experiments a number of modifications to the original LCGU technique were examined, ranging from the use of ^3H -2DG in the place of ^{14}C -2DG to the measurement of LCGU in dissected regions of the mouse brain.

All of the modifications examined here have been successfully used in the literature to measure LCGU, as reviewed in the General Introduction. Having studied first hand the requirements, advantages and disadvantages of each modification, it was possible to select those with which to study the effects of CRF system manipulation on LCGU.

The primary version of the LCGU technique which was used further was intraperitoneal injection of ^3H -2DG in mice, followed by measurement of LCGU in dissected brain regions. The advantages of this version of the technique are the removal of the need for any surgery or stressful intravenous injections, a large reduction in cost (the tritiated ligand is substantially cheaper than the ^{14}C -labelled version, and mice require a ten fold lower dose), and removal of the need for equipment specific to autoradiography. However, the disadvantages of this version of the LCGU technique are an inability to fully quantify LCGU in $\mu\text{mol}/100\text{g}/\text{min}$, an inability to repeatedly measure blood glucose, a reduction in neuroanatomical resolution and the grouping of heterogeneous brain regions into single dissected regions. The inability to fully quantify LCGU removes some ability to standardise measured LCGU to individual animal differences, which could lead to increased variability within treatment groups, and a possible reduction in experimental power. However, a dose of 2DG adjusted to bodyweight was used in an inbred mouse strain to keep differences between individual LCGU measurements to a minimum. In the absence of fully quantitative LCGU measurement, the repeated measurement of blood glucose throughout the LCGU protocol is not essential. However a drawback of measuring only end point blood glucose is that transient changes in blood glucose lasting less than 45 minutes may be missed. The reduction in neuroanatomical resolution introduced by dissecting brain regions makes it impossible to examine the involvement of small regions, either non-dissectable or grouped into larger dissected regions, in changes in LCGU. As such small

regions may contain large amounts of neurons expressing particular neurotransmitters, the opportunity to implicate these particular neurotransmitters in LCGU changes is also lost. Dissection does, however, remove the need for a cryostat and film capturing hardware, which are often limited resources.

This version of the LCGU technique was selected to remove the need for surgically implanted catheters and blood sampling, to limit cost through the use of the tritiated ligand in mice, and to allow the measurement of 2DG retained in the brain without sectioning and autoradiography. However, when drawing conclusions from subsequent studies using this version, it is essential to consider the disadvantages detailed above. The experimental protocol when using this technique in the study of the CRF system and stress is described in detail in the following chapter.

To support any crucial findings in the subsequent studies, it was decided to replicate them using autoradiography with ^{14}C -2DG in mice (intraperitoneal administration), for precise definition of regions of interest using a widely accepted technique. Although not assessed during the studies described in this chapter, this version of the LCGU technique has been widely used in the literature. The use of the more expensive ^{14}C -labelled ligand in these limited cases allows high resolution imaging on film after only 5 days exposure. The advantage of using this version of the LCGU technique to replicate important results is an increase in regional resolution, although the disadvantages of this version are similar to those of the dissection technique, namely, the inability to fully quantify LCGU or to repeatedly measure blood glucose. The experimental protocol when using this technique in subsequent studies is also described in detail in the following chapter.

To allow any effects on the HPA axis, as expected from CRF and related peptides, to be clearly interpreted, it has been confirmed that 2DG does not affect the HPA axis itself at the doses used to measure LCGU.

In order to study the CRF system, the freehand method for ICV injection assessed above was used to centrally administer CRF, the CRF-related peptides, and the peptide CRF₂-selective antagonist antisauvagine-30 (Ruhmann

et al., 1998). The advantage of this technique is the removal of the need for surgical implantation of cannulas, although there are clear disadvantages such as the need to briefly anaesthetise the animal and a degree of stress induced by the injection itself. However, despite these drawbacks, it was possible to measure the effect of CRF in the open field after administration using this technique, and the technique has been used to study the effects of CRF and the Urocortins on behaviour and the HPA axis (Pelleymounter et al., 2000; Pelleymounter et al., 2004). Thus, it was decided to use this non-surgical technique in combination with the non-surgical versions of the LCGU technique described above in using LCGU to study the CRF system, while taking care to consider the drawbacks of both techniques in drawing conclusions from these studies.

Chapter 3 – General Methods

General Methods

In this chapter those methods selected for further use from the previous chapter are described together in detail, as used for the studies in the following chapters. Where required, additional methods are described in the following chapters themselves.

Subjects

Male C57BL/6N mice (Janvier, France) (bodyweight at testing 29-33 g) were individually housed in individually-ventilated cages with food and water available ad libitum, under a standard 12h/12h light-dark cycle (lights on 06:00 a.m.), and at a regulated temperature of $22 \pm 0.5^{\circ}\text{C}$ and humidity of $50 \pm 3\%$. All testing was conducted according to the European Communities Council Directive Nov. 1986 (86/609/EEC) and approved by the animal care and use committee of Johnson & Johnson Pharmaceutical Research and Development. All testing was carried out between 0800 and 1200 hours, i.e. during the early light-phase.

ICV Injections

Peptides were injected ICV using the freehand technique modified from Pellemounter et al. (2000; 2004) as described in the previous chapter. In the modified technique, the mice were weighed, then briefly anaesthetized using isoflurane (4% in 30% O₂, 70% N₂O) until the change of breathing rhythm characteristic of full anaesthesia was apparent. 5 µl of solution was injected using a Hamilton 10 µl syringe and 30-gauge needle limited to 2.5 mm length using a piece of polyethylene tubing. The needle was inserted directly through the skin and skull into the lateral ventricle (see Figure 11a), targeted by visualizing an equilateral triangle between the eyes and center of skull to locate bregma, then inserting the needle 1.5-2 mm laterally to this point (illustrated in Figure 11b). This ensures penetration of the skull at the suture line of the skull plates (Figure 11c) to minimize necessary force, while avoiding the centrally located vein. 5 µl was injected over an approximately 5 sec period, followed by a 10 sec delay to allow diffusion and prevent backflow.

Measurement of local cerebral glucose utilisation (LCGU)

For dissection studies, 2-deoxy-D-[1-³H]glucose (2DG; GE Healthcare, UK) (600 µCi/kg) was injected intraperitoneally immediately after ICV injection. Following 2DG injection, mice were returned to their home-cage for 45 min until decapitation. For the autoradiography studies, 2-deoxy-D-[1-¹⁴C]glucose (GE Healthcare, UK) (167 µCi/kg) was also injected intraperitoneally.

Upon decapitation, trunk blood was collected in BD Microtainer K2E tubes (BD Vacutainer Systems, UK), 10 µl whole blood was used to measure blood glucose using a Lifescan glucose meter (Lifescan Benelux, Belgium), and the remainder was centrifuged at 1100 g for 10 minutes to separate plasma from red blood cells. Plasma samples were collected for measurement of plasma ³H (residual 2DG) and corticosterone and stored at –80°C.

In dissection studies the brains were dissected into frontal cortical area (anterior to corpus callosum), hypothalamus, thalamus, cerebellum, hindbrain (a block defined from the colliculi to the posterior level of the cerebellum), hippocampus and temporal region (including the amygdala). Illustrative diagrams for these dissections can be found in Appendix 1. Tissue samples were weighed, and stored at –80°C until homogenisation.

Brain samples were either homogenized in phosphate-buffered saline (PBS) using glass minibeads and a Mini-BeadBeater-8 (Biospec Products Inc, USA), or dissolved using Solvable tissue solubiliser (Perkin Elmer, Belgium). ³H in plasma and brain samples was measured in duplicate in a TopCount NXT (PerkinElmer, Belgium) microplate scintillation counter after addition of MicroScint-PS (homogenised samples) or Microscint-40 (dissolved samples) scintillant (PerkinElmer, Belgium).

A relative measure of LCGU was calculated as nCi ³H (metabolised 2DG) present per mg of brain tissue. For comparative purposes, percentage change in LCGU was calculated for individual animals against the saline control group mean.

In the autoradiography studies, the brains were rapidly frozen in methylbutane chilled to -35°C on dry ice, and stored at -20°C . 20 μm sections were collected on glass slides using a Leica cryostat (Leica, Germany) and exposed for 5 days on Kodak Biomax MR-1 film (Raytest, Germany) with calibrated standards. LCGU (also defined as nCi/mg tissue) was measured in regions matching those dissected with the addition of the septum and dorsal raphe, using an MCID M1 Digital Densitometry System (Interfocus GmbH, Germany).

Measurement of plasma corticosterone

Plasma corticosterone was measured using ImmuchemTM Double Antibody corticosterone ^{125}I RIA kits (MP Biomedicals, USA).

Statistical Analysis

Statistically small ($n < 30$ per treatment group) data sets may violate the central limit theorem, i.e. are not normally distributed. Statistical tests for normality may also be inaccurate when working with such small data sets. When this is the case, the power of parametric statistics is reduced, and non-parametric analysis may be more suitable. If the Mann-Whitney U test is applied to data which might properly be analysed by the t-test its power increases with sample size and is close to 95 % even for moderate-sized samples. Therefore it is an excellent alternative to the t-test, and does not have the restrictive assumptions and requirements (e.g. normality and equivalent variances) associated with the t-test (Siegel, 1956). Dose-response or single-dose data were statistically analysed non-parametrically using the Kruskal-Wallis (KW) test or the Mann-Whitney (MW) test, respectively (SPSS v13.0, SPSS Belux, Belgium). Where appropriate, post-hoc comparison was performed by the Mann-Whitney test (2-sided). However, there is a lack of non-parametric tests with supported efficiency suitable for the analysis of dual-treatment data, necessitating the use of the parametric 2-way ANOVA for analysis of such data. In dual-treatment (mutant mouse/antagonist pre-treatment studies) studies, data were analysed using 2-way ANOVA (SPSS).

Chapter 4 – The Role of CRF Receptors in the LCGU Response to CRF

The role of the CRF receptors in the LCGU response to CRF

Introduction

As described earlier, corticotropin-releasing factor (CRF) is well known as the key factor which controls the hypothalamic-pituitary-adrenocortical (HPA) axis during basal activity and stress (Vale et al., 1981; Rivier and Plotsky, 1986).

Alterations in CRF activity have been described in a range of neuroendocrine, neurological and psychiatric disorders, including major depressive disorder, post-traumatic stress disorder, schizophrenia and dementia (Holsboer, 1999; Keck and Holsboer, 2001; Steckler, 2005; Steckler and Dautzenberg, 2006).

CRF and its receptors are widely distributed throughout the rodent brain (for overview see Warnock et al., 2006). CRF activates two CRF receptor subtypes, CRF₁ and CRF₂ (Chen et al., 1993; Lovenberg et al., 1995b). CRF₁ has been implicated in the HPA axis response to stress, and in the mediation of stress- and anxiety-related behavior (Steckler and Holsboer, 1999), while a clear role for CRF₂ has yet to be elucidated. Stimulation of CRF₂ has been proposed to attenuate stress-related behaviour (Bale & Vale, 2004; Skelton et al., 2000) but conflicting evidence has also been used to propose a role for CRF₂ in mediating the delayed effects of stress (Takahashi, 2002; plus Steckler, 2005; Steckler & Dautzenberg, 2006 for review).

This series of studies used the LCGU technique to compare the role of the two CRF receptors in neuronal activity. In previous studies, intracerebroventricular (ICV) administration of CRF induced widespread increases in LCGU throughout the brain, including the frontal cortical, hypothalamic, thalamic, cerebellar, midbrain and hindbrain regions (Sharkey et al., 1989; Dube et al., 2000; Freo et al., 2005). None of these studies identified the CRF receptor(s) involved in this response.

In order to isolate the involvement of either CRF receptor in the LCGU response, a combination of the available endogenous peptides and more specific tools were used. Rat/human CRF and human Urocortin 1 were used as non-

specific agonists at both receptors, while human Urocortin 2 and human Stresscopin were used as selective agonists at CRF₂. The role of CRF₁ was investigated using both an antagonist selective for CRF₁, R278995 (Chaki et al., 2004), and mutant mice lacking the CRF₁ receptor. The selective CRF₂ antagonist antisauvagine-30 (Ruhmann et al., 1998) was used to examine the role of this receptor.

Materials and Methods

Subjects

Male C57BL/6N mice were used as described in the General Methods.

Generation of CRF₁ knockout mice

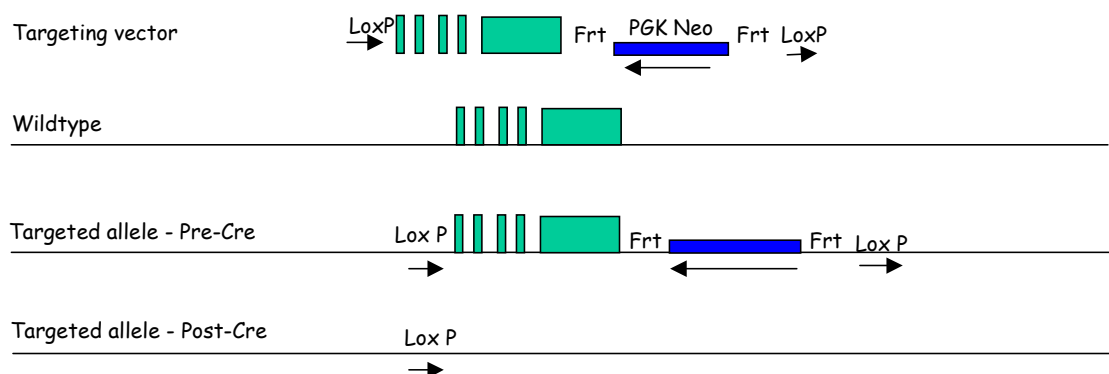
CRF₁ knockout mice (CRF1KO) were obtained from stock in the Johnson & Johnson Pharmaceutical Research & Development transgenic facility. These mutant mice were generated in a similar fashion to those generated by Timpl et al (1998), as outlined below. In these mice the final 5 exons of the CRF₁ receptor gene are deleted, resulting in deletion of the transmembrane regions V, VI and VII, including the G-coupling protein domain and the intracellular cytoplasmic tail (see [ENSEMBL http://www.ensembl.org/Mus_musculus/protview?transcript=ENSMUST00000093925;db=core](http://www.ensembl.org/Mus_musculus/protview?transcript=ENSMUST00000093925;db=core)).

CRF₁ knockout mice were developed in collaboration with Lexicon Genetics Inc.. Using a PCR probe genomic clones were isolated by screening of the 129SvEvBrd derived lambda pKOS genomic library (Wattler et al., 1999). A 9.7 kb genomic clone spanning exon 8 up to the last coding exon 13 was used to generate the targeting vector via yeast-mediated homologous recombination. In this vector a genomic fragment, spanning exon 8 to exon 13, was replaced by a floxed version of exon 8 to exon 13 including a 1.7 kb PGK-neo selection cassette flanked by two Frt sites. The NotI-linearized vector was electroporated into 129 Sv/Evbrd(LEX1) embryonic stem (ES) cells and G418-fialuridine (FIAU)-resistant ES cell clones were isolated and analysed for homologous recombination by Southern blot analysis. Targeted ES cell clones were injected

into C57BL/6(albino) blastocysts, and the resulting chimeras were mated to C57BL/6(albino) females to generate animals heterozygote for the floxed CRF₁ allele. These were subsequently crossed to Protamine-Cre mice (O'Gorman et al., 1997) and male descendants heterozygote for both the floxed CRF₁ allele and the Protamine Cre transgene were crossed to C57BL/6 females to obtain heterozygote CRF₁ knockout animals. These were subsequently crossed to generate homozygote knockout, heterozygote knockout and wild-type littermate animals with an overall C57BL/6 background. The mutant generation process is illustrated in Figure 26. Sufficient breeding cycles to generate the required experimental animal numbers are necessary due an increased prenatal lethality in homozygote CRF₁ knockout animals. The expression of CRF₁ and CRF₂ receptor mRNA in mutant animals was assessed by quantitative PCR (for method and results see Appendix 2).

For LCGU studies only homozygote knockout males and male wild-type littermate controls were used.

Figure 26: Illustration of the targeting vector, subsequent allele and resultant allele for generation of CRF₁ knockout mice. The targeting vector includes LoxP sites and PGK-neo selection cassette for removal of exons 8 to 12 by recombinase in Protamine-Cre mice.



Peptides and ICV Injections

Peptides were injected ICV using the freehand technique validated and described earlier.

Rat/human CRF (0.1-1 µg), human Urocortin 1 (1, 3 µg), human Urocortin 2 (1, 10 µg), human Stresscopin (0.1, 1, 10 µg) (Bachem, Germany) and antisauvagine-30 (1-5 µg) were dissolved in 0.9% saline and injected in a volume of 5 µl. Peptide doses selected were based on those reported to elicit behavioural responses (Butler et al., 1990; Dunn and Berridge, 1990; Jones et al., 1998; Benoit et al., 2000; Valdez et al., 2002; Valdez et al., 2003).

For the study combining CRF and antisauvagine-30, the two peptides were injected as a mixture dissolved in 0.9% saline and injected in a volume of 5 µl. The injection of these peptides as a mixture has been shown to produce effective antagonism in a behavioural model of anxiety-related behaviour (Risbrough et al., 2003).

Compounds

R278995 is a highly CRF₁ –selective antagonist co-developed by Johnson & Johnson Pharmaceutical Research & Development and Taisho Pharmaceuticals (Japan) with demonstrated pharmacokinetics and pharmacological activity (Chaki et al., 2004). Perhaps of note, R278995 also has a high affinity for σ 1 receptors, which may also have a role in anxiety and depression, although studies suggested that these receptors were unlikely to be involved in the effects of R278995 (Chaki et al., 2004). In initial studies it appeared that after a preinjection (vehicle or compound) the LCGU response to CRF in the positive control group was no longer present, making it impossible to study the effect of R278995 on this response via this method. This disruption may represent the effect of a mild pre-stressor on the subsequent response to CRF, which may itself be of interest for future studies. However, in order to study the effect of R278995 on the acute effects of CRF on LCGU, it was decided to administer the compound through the diet, thus ensuring the presence of R278995 in the animal without the need for an injection prior to the ICV administration of CRF and 2DG injection.

The efficiency of dietary administration was assessed in a series of pilot studies. The data from these pilot studies is presented in Appendix 3.

The dry compound was administered in a powdered premixed diet from Research Diets (<http://www.researchdiets.com>) to an approximate dose of 40 mg/kg/day based on mice consuming approximately 4 grams per 24 hours (as indicated by pilot studies). The final diet was D12450B. In both pilot and final studies the powdered diet replaced the standard diet for 5 days prior to testing. In the pilot studies the plasma and brain levels of R278995 were measured by Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC-MS/MS) (method in Appendix 3) on day 6 at three time points to assess the fall in levels based upon the 1.8 hour plasma half-life of the compound, and also taking into consideration that food consumption effectively stops during the light-phase. These time points were selected to examine levels of R278995 at times relevant to the measurement of LCGU (i.e. during the early light-phase when all LCGU studies were carried out). The occupancy of CRF₁ receptors in the frontal cortex was also measured on day 6, corresponding to occupancy at 0800 and 1100 hours (in the early light-phase to match LCGU studies). Both plasma/brain compound levels and CRF₁ occupancy were compared to those after acute oral administration of 40 mg/kg R278995 (1 hour preinjection).

These pilot studies confirmed that the compound reaches the plasma and brain following administration in the diet, and CRF₁ occupancy immediately after the dark phase reached a similar level to acute administration (82.3 % acute versus 72.4 % dietary). Thus, dietary administration of R278995 was used in a full scale study to examine the role of CRF₁ in the acute LCGU response to CRF.

Measurement of local cerebral glucose utilisation (LCGU) and plasma corticosterone

LCGU and plasma corticosterone were measured as described in the General Methods. In order to match the times with optimum occupancy of CRF₁ receptors with R278995, based on the studies described in Appendix 3, all testing was carried out between 0800 and 1200 hours, i.e. during the early light-phase.

Statistical Analysis

Data were analysed as described in the General Methods. Briefly, multiple comparisons were made using the Kruskal-Wallis test, followed where appropriate by the Mann-Whitney-U test for post-hoc comparisons. Non-parametric tests were used to account for the possibility that data of this type does not meet the central limit theorem. For mutant mouse studies or those with dual treatment, two-way ANOVA was used.

Results

Local Cerebral Glucose Utilisation

Non-specific CRF_{1/2} agonists CRF & Urocortin 1

In dissected brain regions, CRF increased LCGU in a dose-dependent manner in hypothalamic, thalamic, cerebellar and hippocampal areas (Table 4; Figure 27). The largest response to CRF was 67 ± 18 % at the 1 μ g dose in the cerebellar region. A significant increase was seen following the 0.5 μ g dose in the hypothalamic sample ($p < 0.01$). Similar tendencies were observed at thalamic ($p = 0.072$), hippocampal ($p = 0.054$) and cerebellar ($p = 0.054$) levels. A dose of 1 μ g CRF increased LCGU at thalamic ($p < 0.01$), hippocampal ($p = 0.038$) and cerebellar ($p = 0.011$) levels, and a similar tendency was seen in the hypothalamus ($p = 0.054$).

When measured by autoradiography, 0.5 μ g CRF significantly increased LCGU in a number of regions matching those affected in the dissection study. LCGU was significantly increased in septal ($p = 0.017$), hypothalamic ($p = 0.030$) hippocampal ($p = 0.004$), and cerebellar ($p = 0.017$) regions, with a maximal CRF-induced effect of 51 ± 8 % in the hippocampus (Table 5; Figure 28; representative autoradiographs shown in Figure 29). Similar trends for increased LCGU were seen in frontal cortical ($p = 0.082$), amygdala ($p = 0.063$) and hindbrain ($p = 0.052$) regions. However, in contrast to the dissection study, no trend-like effect on LCGU after CRF treatment was seen in the thalamus at the corresponding dose of 0.5 μ g. (Note: a higher dose of CRF was not tested by autoradiography as this dose corresponded to an original single dose of CRF tested by dissection. The dissection study was later expanded to a dose-response).

Table 4: Changes in LCGU (measured by region dissection) from baseline (mean \pm SEM) after ICV administration of CRF (Saline n=7, 0.1 μ g n=7, 0.5 μ g n=8, 1 μ g n=7). *p<0.05, **p<0.01, (*)p<0.1. (Temporal region dissected to include the amygdala).

CRF	LCGU (nCi/mg)			
Region	Saline	0.1 μ g	0.5 μ g	1.0 μ g
Frontal Cortical	0.12 \pm 0.02	0.09 \pm 0.02	0.13 \pm 0.01	0.14 \pm 0.01
Hypothalamus	0.12 \pm 0.01	0.12 \pm 0.02	0.17 \pm 0.01 **	0.16 \pm 0.02 (*)
Thalamus	0.18 \pm 0.03	0.16 \pm 0.03	0.24 \pm 0.02 (*)	0.28 \pm 0.02 **
Cerebellum	0.11 \pm 0.02	0.12 \pm 0.02	0.17 \pm 0.02 (*)	0.19 \pm 0.02 *
Hindbrain	0.08 \pm 0.01	0.08 \pm 0.01	0.10 \pm 0.01	0.11 \pm 0.01
Hippocampus	0.10 \pm 0.01	0.10 \pm 0.02	0.13 \pm 0.01 (*)	0.15 \pm 0.01 *
Temporal	0.12 \pm 0.02	0.10 \pm 0.02	0.12 \pm 0.01	0.14 \pm 0.01

CRF	% change in LCGU		
Region	0.1 μ g	0.5 μ g	1.0 μ g
Frontal Cortical	-19 \pm 13	10 \pm 8	18 \pm 7
Hypothalamus	-3 \pm 15	37 \pm 7	35 \pm 14
Thalamus	-10 \pm 16	36 \pm 14	59 \pm 10
Cerebellum	3 \pm 19	45 \pm 16	67 \pm 18
Hindbrain	-3 \pm 17	26 \pm 12	45 \pm 14
Hippocampus	-1 \pm 16	30 \pm 12	51 \pm 12
Temporal	-13 \pm 16	4 \pm 8	14 \pm 7

Figure 27: Changes in LCGU (measured by region dissection) from baseline (mean \pm SEM) after ICV administration of CRF (Saline n=7, 0.1 μ g n=7, 0.5 μ g n=8, 1 μ g n=7). *p<0.05, **p<0.01, (*)p<0.1. (Region abbreviations match those used in Figure 12).

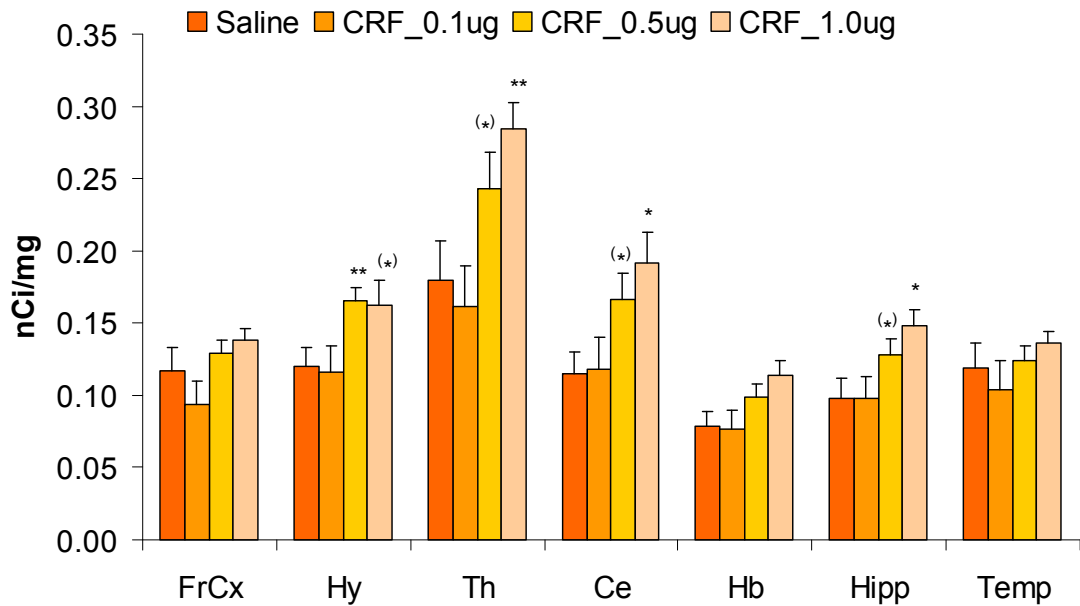


Table 5: Changes in LCGU (measured by autoradiography) from baseline (mean \pm SEM) after ICV administration of CRF (Saline n=5, CRF 0.5 μ g n=6). *p<0.05, **p<0.01, (*)p<0.1.

CRF	rLCGU (nCi/mg)		% change in rLCGU
Region	Saline	0.5 μ g	0.5 μ g
Frontal Cortical	1.07 \pm 0.06	1.22 \pm 0.08 (*)	14 \pm 7
Septum	0.82 \pm 0.08	1.00 \pm 0.03 *	23 \pm 3
Striatum	1.17 \pm 0.10	1.36 \pm 0.06	16 \pm 6
Hypothalamus	0.64 \pm 0.09	0.89 \pm 0.05 *	39 \pm 8
Thalamus	1.26 \pm 0.09	1.44 \pm 0.07	14 \pm 5
Amygdala	0.69 \pm 0.11	1.02 \pm 0.06 (*)	47 \pm 9
Hippocampus	0.69 \pm 0.08	1.04 \pm 0.06 **	51 \pm 8
Cerebellum	1.01 \pm 0.11	1.34 \pm 0.07 *	34 \pm 7
Hindbrain	1.02 \pm 0.11	1.24 \pm 0.07 (*)	22 \pm 6

Figure 28: Changes in LCGU (measured by autoradiography) from baseline (mean \pm SEM) after ICV administration of CRF (Saline n=5, CRF 0.5 μ g n=6). *p<0.05, **p<0.01, (*)p<0.1. (Additional abbreviations: Sept – septum, Cpu – caudate putamen/striatum, Amyg – amygdala).

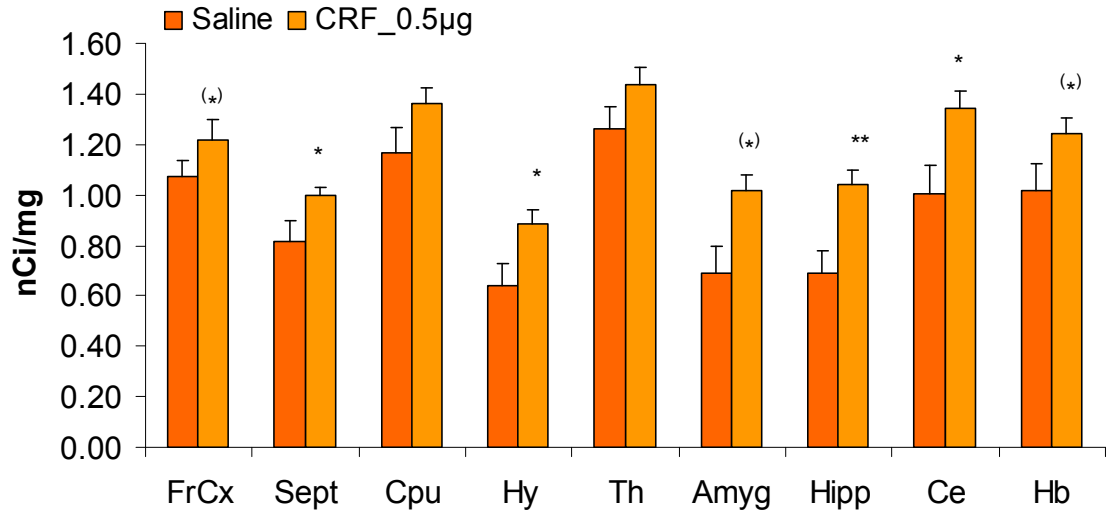
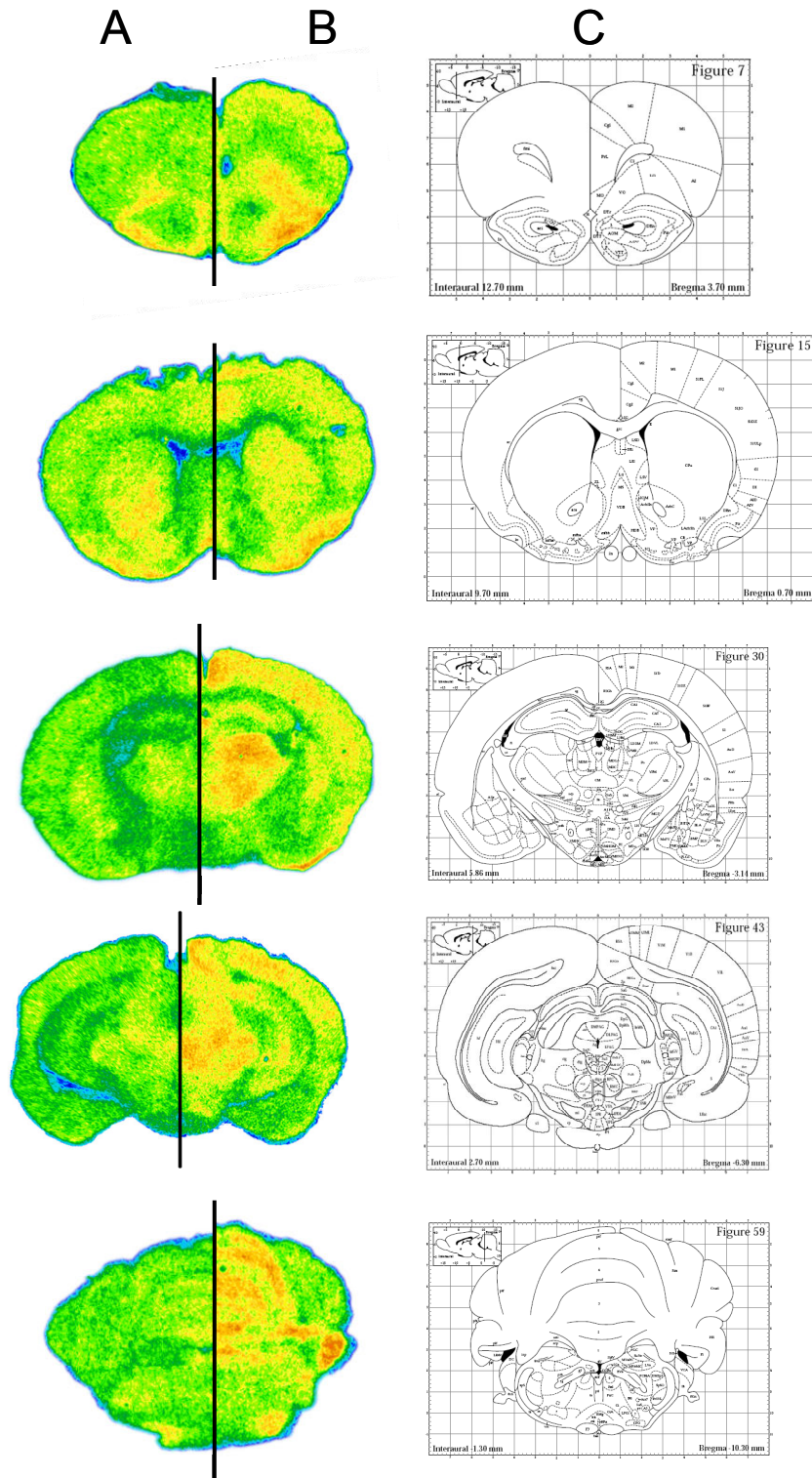


Figure 29: Split-hemispheric comparisons of representative autoradiographs of 20 μ m coronal sections from (A) ICV saline-treated and (B) 0.5 μ g ICV CRF-treated mice (C) Representative figures from Paxinos & Watson atlas at this level. (false colour temperature scale, warmer corresponds to increased LCGU).

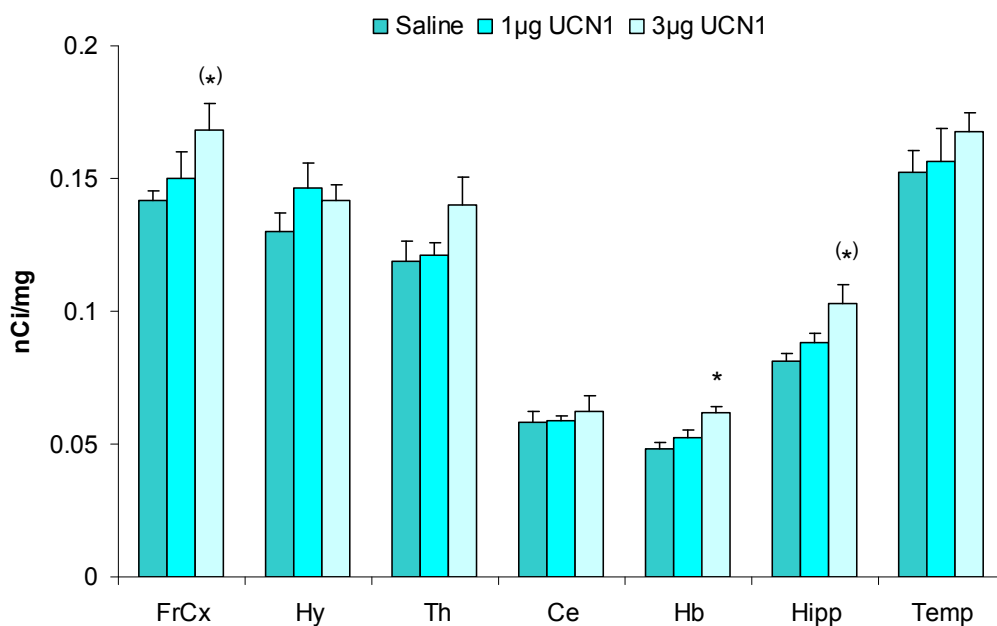


Urocortin 1 (UCN1) dose-dependently increased LCGU in a number of brain regions, though to a lesser extent than CRF. The largest increase, 27 ± 6 %, was seen in the dissected hindbrain region (Table 6; Figure 30), in which CRF only induced a trend-like effect, detected only in the autoradiography study. LCGU was significantly increased at the 3 μ g dose ($p=0.005$) in the hindbrain, with similar trends towards increased LCGU in frontal cortical and hippocampal regions (KW $p=0.079$ and 0.052 , respectively).

Table 6: Changes in LCGU from baseline (mean \pm SEM) after ICV administration of Urocortin 1 (Saline $n=7$, 1 μ g $n=5$, 3 μ g $n=5$). * $p<0.05$, (*) $p<0.1$.

Urocortin 1 Region	Saline	LCGU (nCi/mg)			% change in LCGU	
		1 μ g	3 μ g		1 μ g	3 μ g
Frontal Cortical	0.14 \pm 0.00	0.15 \pm 0.01	0.17 \pm 0.01	(*)	6 \pm 7	19 \pm 7
Hypothalamus	0.13 \pm 0.01	0.15 \pm 0.01	0.14 \pm 0.01		13 \pm 7	9 \pm 5
Thalamus	0.12 \pm 0.01	0.12 \pm 0.00	0.14 \pm 0.01		2 \pm 4	18 \pm 9
Cerebellum	0.06 \pm 0.00	0.06 \pm 0.00	0.06 \pm 0.01		0 \pm 4	7 \pm 11
Hindbrain	0.05 \pm 0.00	0.05 \pm 0.00	0.06 \pm 0.00	*	9 \pm 5	27 \pm 6
Hippocampus	0.08 \pm 0.00	0.09 \pm 0.00	0.10 \pm 0.01	(*)	9 \pm 5	27 \pm 9
Temporal	0.15 \pm 0.01	0.16 \pm 0.01	0.17 \pm 0.01		3 \pm 8	10 \pm 5

Figure 30: Changes in LCGU from baseline (mean \pm SEM) after ICV administration of Urocortin 1 (Saline $n=7$, 1 μ g $n=5$, 3 μ g $n=5$). * $p<0.05$, (*) $p<0.1$.



Role of CRF₁ and CRF₂ in the LCGU response to CRF

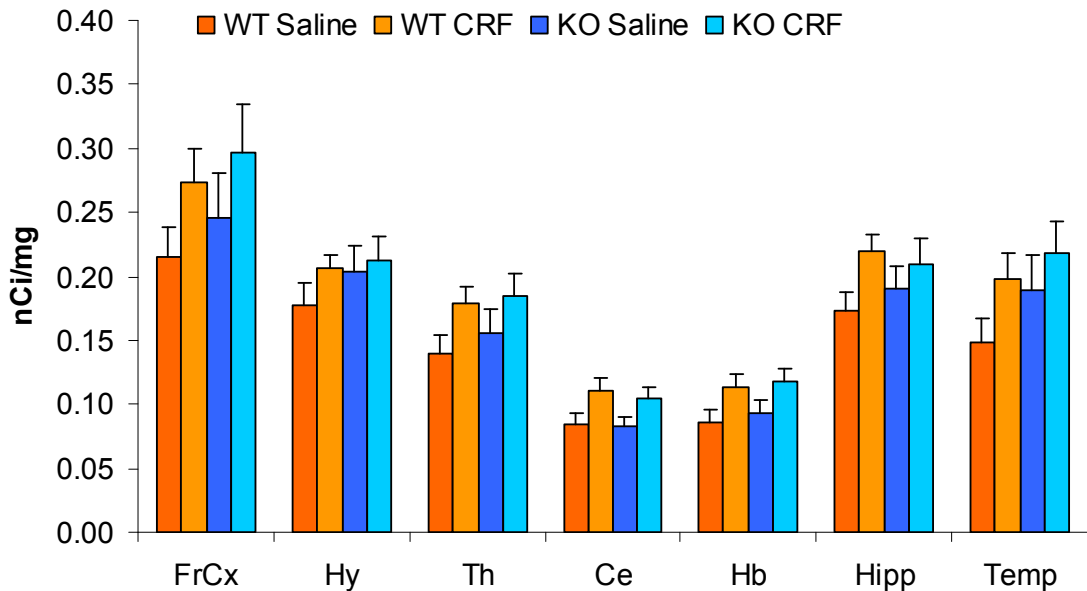
To examine the involvement of either CRF₁ or CRF₂ receptors in the LCGU response to CRF, a combination of selective antagonists and a mutant mouse model were used. To isolate the involvement of CRF₁, the effect of CRF on LCGU was measured in both mice lacking the CRF₁ receptor (CRF1KO), and after pretreatment with the selective CRF₁ antagonist R278995. To study CRF₂, the selective antagonist antisauvagine-30 was co-administered with CRF. In these studies LCGU was measured in dissected brain regions.

The LCGU response to 0.5 µg CRF was unaltered in mice lacking the CRF₁ receptor compared to their wild-type littermates. LCGU was significantly increased in thalamic (2-way ANOVA treatment effect, $F_{1,27}=4.432$, $p=0.045$), cerebellum ($F_{1,27}=7.638$, $p=0.010$), hindbrain ($F_{1,27}=5.880$, $p=.022$) and hippocampal ($F_{1,27}=4.332$, $p=0.047$) regions (Table 7; Figure 31). No significant 2-way ANOVA interaction effect between CRF1KO and CRF treatment was found.

Table 7: Changes in LCGU from baseline (mean ± SEM) after ICV administration of 0.5 µg CRF in mice lacking the CRF₁ receptor (CRF1KO) compared to wild-type controls (WT) (WT+Saline n=8, WT+CRF n=8, KO+Saline n=8, KO+CRF n=7).

CRF in CRF1KO Region	LCGU (nCi/mg)				% change in LCGU	
	WT+Saline	WT+CRF	KO+Saline	KO+CRF	WT+CRF	KO+CRF
Frontal Cortical	0.22 ± 0.02	0.27 ± 0.03	0.25 ± 0.04	0.30 ± 0.04	27 ± 12	21 ± 16
Hypothalamus	0.18 ± 0.02	0.21 ± 0.01	0.20 ± 0.02	0.21 ± 0.02	16 ± 6	5 ± 10
Thalamus	0.14 ± 0.02	0.18 ± 0.01	0.16 ± 0.02	0.19 ± 0.02	29 ± 9	19 ± 11
Cerebellum	0.08 ± 0.01	0.11 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	31 ± 11	25 ± 11
Hindbrain	0.09 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	31 ± 13	26 ± 12
Hippocampus	0.17 ± 0.01	0.22 ± 0.01	0.19 ± 0.02	0.21 ± 0.02	27 ± 8	10 ± 10
Temporal	0.15 ± 0.02	0.20 ± 0.02	0.19 ± 0.03	0.22 ± 0.02	33 ± 13	16 ± 13

Figure 31: Changes in LCGU from baseline (mean \pm SEM) after ICV administration of 0.5 μ g CRF in mice lacking the CRF₁ receptor (CRF1KO) compared to wild-type controls (WT) (WT+Saline n=8, WT+CRF n=8, KO+Saline n=8, KO+CRF n=7).



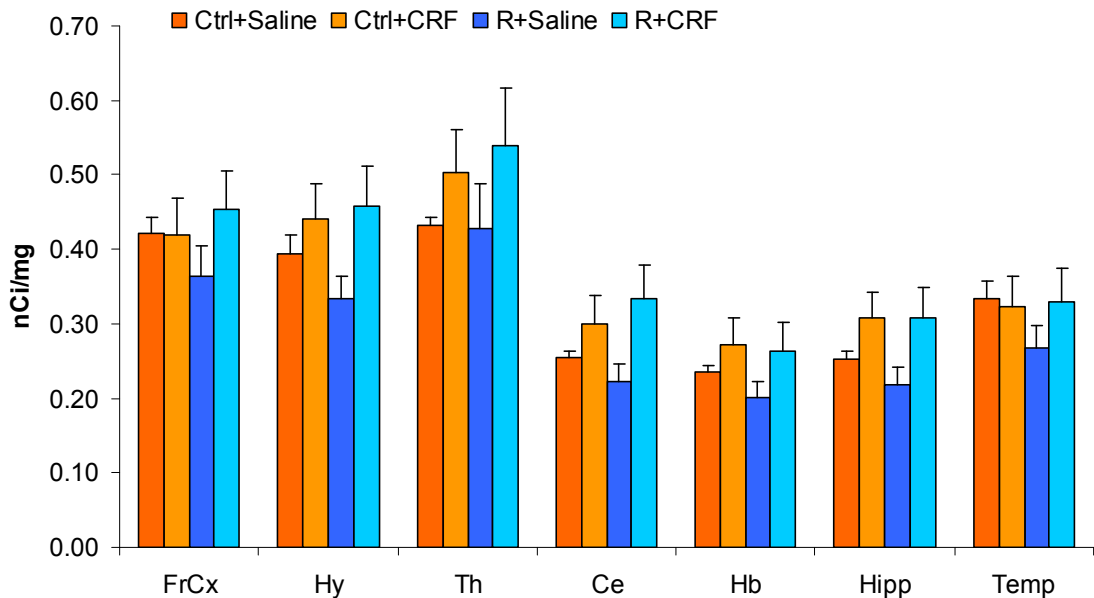
In agreement with the data from CRF1KO mice, the CRF₁ antagonist R278995 had no effect on the LCGU response to 1 μ g CRF (Table 8; Figure 32). CRF significantly increased LCGU in both animals that received control diet and animals that received compound-treated diet in hypothalamic (2-way ANOVA treatment effect, $F_{1,28}=4.582$, $p=0.041$), cerebellum ($F_{1,28}=5.942$, $p=.021$) and hippocampal ($F_{1,28}=6.537$, $p=.016$) regions. No significant 2-way ANOVA interaction effect between R278995 and CRF treatment was found.

Table 8: The effect of R278995 (40 mg/kg/day, 5 days) on changes in LCGU from baseline (mean \pm SEM) after ICV administration of 1 μ g CRF (all treatment groups n=8). * 2-way ANOVA treatment effect $p < 0.05$.

R278995/CRF Region	LCGU (nCi/mg)			
	Ctrl+Saline	Ctrl+CRF	R+Saline	R+CRF
Frontal Cortical	0.42 \pm 0.02	0.42 \pm 0.05	0.36 \pm 0.04	0.45 \pm 0.05
Hypothalamus	0.39 \pm 0.03	0.44 \pm 0.05	0.33 \pm 0.03	0.46 \pm 0.05
Thalamus	0.43 \pm 0.01	0.50 \pm 0.06	0.43 \pm 0.06	0.54 \pm 0.08
Cerebellum	0.25 \pm 0.01	0.30 \pm 0.04	0.22 \pm 0.03	0.33 \pm 0.04
Hindbrain	0.23 \pm 0.01	0.27 \pm 0.04	0.20 \pm 0.02	0.26 \pm 0.04
Hippocampus	0.25 \pm 0.01	0.31 \pm 0.03	0.22 \pm 0.02	0.31 \pm 0.04
Temporal	0.33 \pm 0.02	0.32 \pm 0.04	0.27 \pm 0.03	0.33 \pm 0.04

R278995/CRF Region	% change in LCGU		
	Ctrl+CRF	R+Saline	R+CRF
Frontal Cortical	0 \pm 12	-13 \pm 10	8 \pm 13
Hypothalamus	12 \pm 12	-15 \pm 8	16 \pm 13
Thalamus	16 \pm 14	-1 \pm 14	25 \pm 18
Cerebellum	18 \pm 15	-13 \pm 10	32 \pm 18
Hindbrain	16 \pm 15	-14 \pm 9	12 \pm 16
Hippocampus	22 \pm 13	-13 \pm 9	22 \pm 16
Temporal	-4 \pm 12	-20 \pm 9	-1 \pm 13

Figure 32: The effect of R278995 (40 mg/kg/day, 5 days) on changes in LCGU from baseline (mean \pm SEM) after ICV administration of 1 μ g CRF (all treatment groups n=8).



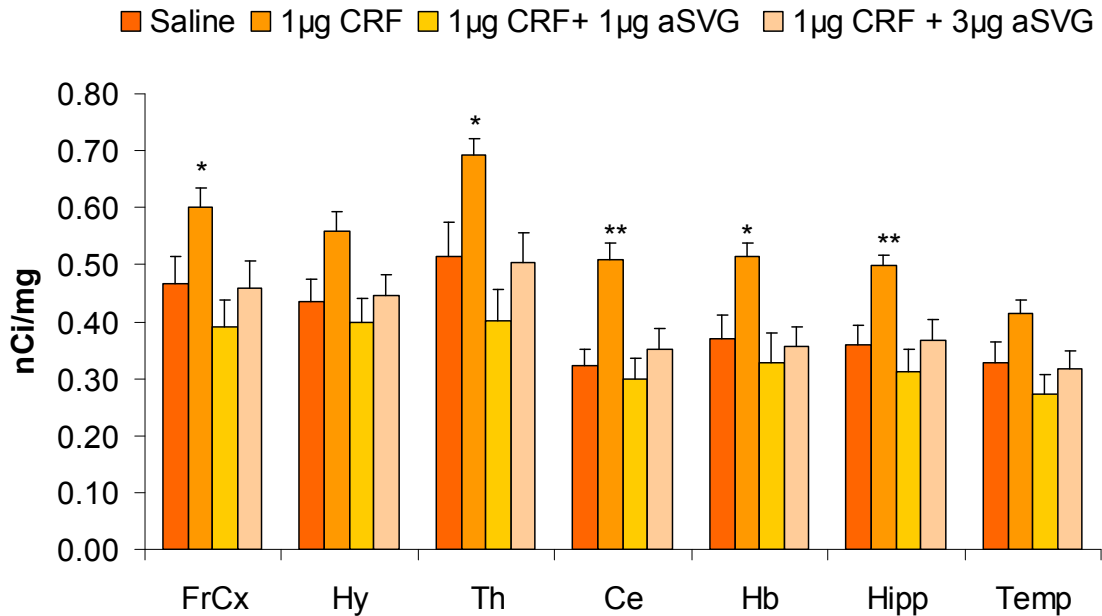
In contrast to the continued effect of CRF on LCGU in CRF1KO mice and after treatment with R278995, the CRF₂-selective antagonist antisauvagine-30 completely abolished the LCGU to CRF, at both doses tested (Table 9; Figure 33). Treatment with 1 μ g CRF alone significantly increased LCGU in thalamic ($p=0.029$), cerebellum ($p=0.001$) and hippocampal ($p=0.005$) regions as reported earlier, with the addition of the frontal cortical region ($p=0.029$) and hindbrain ($p=0.013$). This response was completely abolished by 1 or 3 μ g antisauvagine-30 administered together with 1 μ g CRF.

Table 9: The effect of antisauvagine-30 (aSVG) (1 and 3 μ g) on changes in LCGU from baseline (mean \pm SEM) after ICV administration of 1 μ g CRF (Saline n=8, 1 μ g CRF n=6, 1 μ g CRF + 3 μ g aSVG n=8, 1 μ g CRF + 3 μ g aSVG n=8). *p<0.05, **p<0.01.

aSVG vs. CRF		LCGU (nCi/mg)			
Region	Saline	CRF	1 μ g aSVG	3 μ g aSVG	
Frontal Cortical	0.47 \pm 0.05	0.60 \pm 0.03 *	0.39 \pm 0.05	0.46 \pm 0.05	
Hypothalamus	0.43 \pm 0.04	0.56 \pm 0.03	0.40 \pm 0.04	0.45 \pm 0.04	
Thalamus	0.51 \pm 0.06	0.69 \pm 0.03 *	0.40 \pm 0.05	0.50 \pm 0.05	
Cerebellum	0.32 \pm 0.03	0.51 \pm 0.03 **	0.30 \pm 0.04	0.35 \pm 0.04	
Hindbrain	0.37 \pm 0.04	0.51 \pm 0.02 *	0.33 \pm 0.05	0.36 \pm 0.03	
Hippocampus	0.36 \pm 0.04	0.50 \pm 0.02 **	0.31 \pm 0.04	0.37 \pm 0.04	
Temporal	0.33 \pm 0.04	0.41 \pm 0.02	0.27 \pm 0.03	0.32 \pm 0.03	

aSVG vs. CRF		% change in LCGU	
Region	CRF	1 μ g aSVG	3 μ g aSVG
Frontal Cortical	29 \pm 8	-16 \pm 10	-1 \pm 10
Hypothalamus	29 \pm 8	-8 \pm 10	3 \pm 8
Thalamus	35 \pm 6	-22 \pm 10	-2 \pm 10
Cerebellum	58 \pm 9	-7 \pm 12	9 \pm 11
Hindbrain	39 \pm 6	-12 \pm 14	-4 \pm 9
Hippocampus	39 \pm 5	-13 \pm 11	2 \pm 10
Temporal	26 \pm 7	-17 \pm 10	-3 \pm 9

Figure 33: The effect of antisauvagine-30 (aSVG) (1 and 3 μ g) on changes in LCGU from baseline (mean \pm SEM) after ICV administration of 1 μ g CRF (Saline n=8, 1 μ g CRF n=6, 1 μ g CRF + 3 μ g aSVG n=8, 1 μ g CRF + 3 μ g aSVG n=8). *p<0.05, **p<0.01.

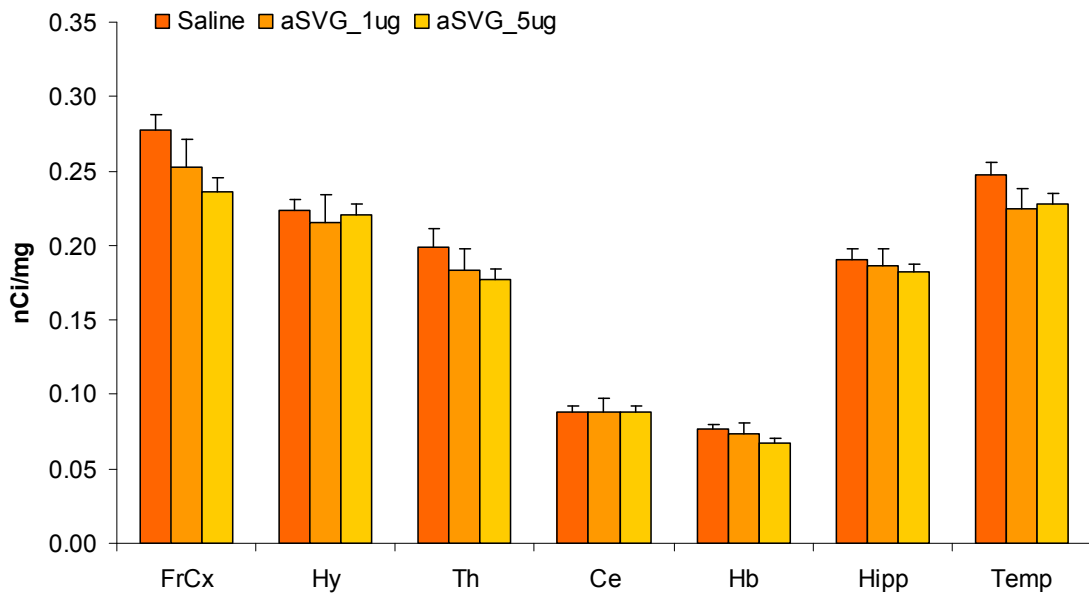


When administered alone, antisauvagine-30 had no effect on LCGU at the dose of either 1 or 5 μ g (Table 10; Figure 34).

Table 10: The effect of antisauvagine-30 (aSVG) (1 and 5 μ g) on LCGU from baseline (mean \pm SEM) (Saline n=8, 1 μ g aSVG n=8, 5 μ g aSVG n=9).

aSVG Region	LCGU (nCi/mg)			% change in LCGU	
	Saline	1ug	5ug	1 μ g	5 μ g
Frontal Cortical	0.28 \pm 0.01	0.25 \pm 0.02	0.24 \pm 0.01	-9 \pm 7	-15 \pm 5
Hypothalamus	0.22 \pm 0.01	0.22 \pm 0.02	0.22 \pm 0.01	-4 \pm 8	-1 \pm 6
Thalamus	0.20 \pm 0.01	0.18 \pm 0.01	0.18 \pm 0.01	-7 \pm 7	-11 \pm 5
Cerebellum	0.09 \pm 0.00	0.09 \pm 0.01	0.09 \pm 0.01	-1 \pm 12	0 \pm 7
Hindbrain	0.08 \pm 0.00	0.07 \pm 0.01	0.07 \pm 0.00	-4 \pm 10	-12 \pm 5
Hippocampus	0.19 \pm 0.01	0.19 \pm 0.01	0.18 \pm 0.01	-2 \pm 6	-5 \pm 5
Temporal	0.25 \pm 0.01	0.22 \pm 0.01	0.23 \pm 0.01	-9 \pm 6	-8 \pm 6

Figure 34: The effect of antisauvagine-30 (aSVG) (1 and 5 μ g) on LCGU from baseline (mean \pm SEM) (Saline n=8, 1 μ g aSVG n=8, 5 μ g aSVG n=9).



In contrast to the effects on LCGU induced by CRF and UCN1, ICV administration of the selective CRF₂ agonists Urocortin 2 (UCN2) (Table 11; Figure 35) and Stresscopin (SCP) (Table 12; Figure 36) had no effect on LCGU.

Table 11: The effect of Urocortin 2 (UCN2) (1 and 10 μ g) on LCGU from baseline (mean \pm SEM) (Saline n=11, 1 μ g UCN2n=11, 10 μ g UCN2n=10).

Urocortin 2 Region	LCGU (nCi/mg)			% change in LCGU	
	Saline	1 μ g	10 μ g	1 μ g	10 μ g
Frontal Cortical	0.17 \pm 0.02	0.17 \pm 0.01	0.13 \pm 0.01	0 \pm 9	-19 \pm 9
Hypothalamus	0.15 \pm 0.02	0.15 \pm 0.02	0.12 \pm 0.01	1 \pm 11	-20 \pm 9
Thalamus	0.13 \pm 0.02	0.14 \pm 0.01	0.10 \pm 0.01	6 \pm 8	-22 \pm 10
Cerebellum	0.07 \pm 0.01	0.07 \pm 0.01	0.06 \pm 0.01	-4 \pm 9	-17 \pm 11
Hindbrain	0.07 \pm 0.01	0.07 \pm 0.01	0.06 \pm 0.01	7 \pm 9	-16 \pm 12
Hippocampus	0.15 \pm 0.02	0.15 \pm 0.01	0.13 \pm 0.01	3 \pm 8	-16 \pm 10
Temporal	0.18 \pm 0.02	0.19 \pm 0.02	0.15 \pm 0.02	3 \pm 9	-20 \pm 9

Figure 35: The effect of Urocortin 2 (UCN2) (1 and 10 μ g) on LCGU from baseline (mean \pm SEM) (Saline n=11, 1 μ g UCN2n=11, 10 μ g UCN2n=10).

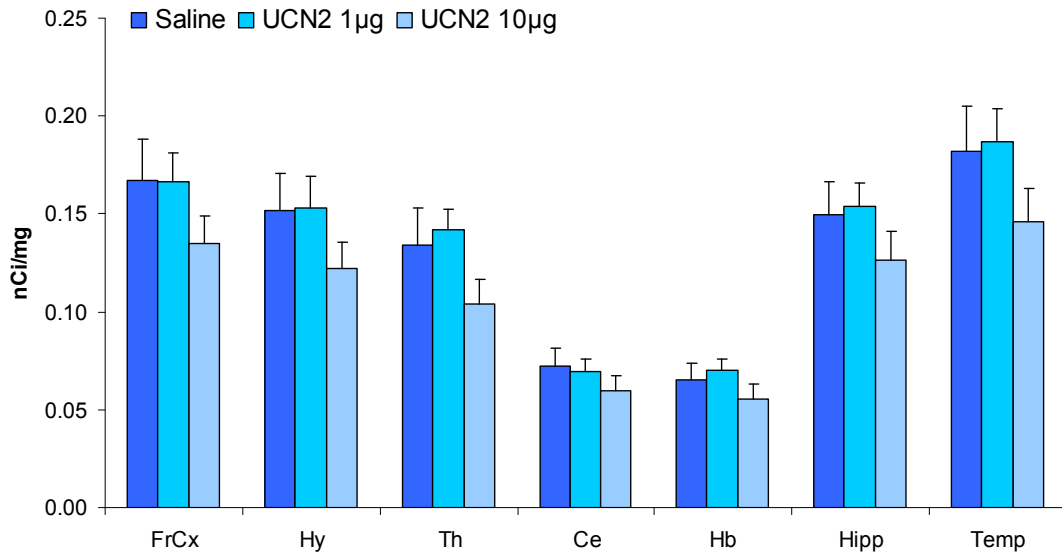
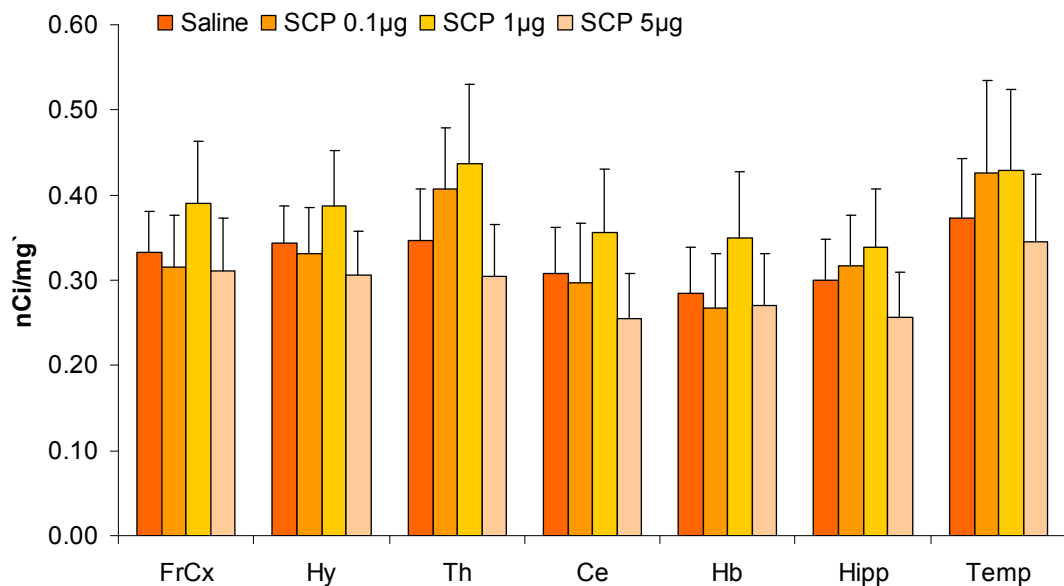


Table 12: The effect of Stresscopin (SCP) (0.1, 1 and 5 μ g) on LCGU from baseline (mean \pm SEM) (Saline n=8, 0.1 μ g SCP n=8, 1 μ g SCP n=8, 5 μ g SCP n=7).

Stresscopin Region	LCGU (nCi/mg)			
	saline	0.1 μ g	1 μ g	5 μ g
Frontal Cortical	0.33 \pm 0.05	0.31 \pm 0.06	0.39 \pm 0.07	0.31 \pm 0.06
Hypothalamus	0.34 \pm 0.04	0.33 \pm 0.05	0.39 \pm 0.07	0.31 \pm 0.05
Thalamus	0.35 \pm 0.06	0.41 \pm 0.07	0.44 \pm 0.09	0.31 \pm 0.06
Cerebellum	0.31 \pm 0.06	0.30 \pm 0.07	0.36 \pm 0.07	0.25 \pm 0.05
Hindbrain	0.28 \pm 0.05	0.27 \pm 0.06	0.35 \pm 0.08	0.27 \pm 0.06
Hippocampus	0.30 \pm 0.05	0.32 \pm 0.06	0.34 \pm 0.07	0.26 \pm 0.05
Temporal	0.37 \pm 0.07	0.43 \pm 0.11	0.43 \pm 0.10	0.34 \pm 0.08

Stresscopin Region	% change in LCGU		
	0.1 μ g	1 μ g	5 μ g
Frontal Cortical	-5 \pm 18	17 \pm 22	-6 \pm 19
Hypothalamus	-3 \pm 16	13 \pm 19	-11 \pm 15
Thalamus	18 \pm 21	26 \pm 27	-12 \pm 17
Cerebellum	-4 \pm 23	16 \pm 24	-17 \pm 17
Hindbrain	-6 \pm 23	23 \pm 27	-5 \pm 21
Hippocampus	6 \pm 20	13 \pm 23	-14 \pm 18
Temporal	14 \pm 29	15 \pm 26	-8 \pm 21

Figure 36: The effect of Stresscopin (SCP) (0.1, 1 and 5 μg) on LCGU from baseline (mean \pm SEM) (Saline $n=8$, 0.1 μg SCP $n=8$, 1 μg SCP $n=8$, 5 μg SCP $n=7$).



Based on the previous studies with CRF and antisauvagine-30, the lack of effect of these CRF₂-selective agonists was unexpected. Therefore, the effects of Stresscopin on LCGU were studied further, by autoradiography, and in CRF1KO mice. Despite its relatively lower affinity for CRF₂ (see Table 2), Stresscopin was chosen for these further studies as time-dependent behavioural effects have been reported for Urocortin 2 (Valdez et al., 2002), complicating the timing of injection before measuring LCGU. The delayed effects of Urocortin 2 in vivo may reflect its lack of a consensus site for proteolytic cleavage (Hauger et al., 2003). Stresscopin has not been reported to lack such a site, and direct behavioural effects after administration have been reported (Valdez et al., 2003), although the possibility of further time-dependent effects cannot be fully excluded.

0.1 μg Stresscopin had no effect on LCGU measured by autoradiography (Table 13; Figure 37), and had no effect on LCGU in mice lacking the CRF₁ receptor or their wild-type littermates (Table 14; Figure 38).

Table 13: The effect of Stresscopin (SCP) (0.1 μ g) on LCGU (measured by autoradiography) from baseline (mean \pm SEM) (Saline n=12, 0.1 μ g SCP n=11).

Stresscopin	LCGU (nCi/mg)		% change in LCGU
Region	Saline	0.1 μ g	0.1 μ g
Frontal Cortical	0.95 \pm 0.06	1.03 \pm 0.07	9 \pm 7
Septum	0.70 \pm 0.05	0.74 \pm 0.06	6 \pm 8
Striatum	1.04 \pm 0.07	1.15 \pm 0.08	10 \pm 8
Hypothalamus	0.58 \pm 0.04	0.68 \pm 0.05	17 \pm 8
Thalamus	1.07 \pm 0.07	1.20 \pm 0.08	13 \pm 8
Amygdala	0.66 \pm 0.05	0.79 \pm 0.06	20 \pm 9
Hippocampus	0.65 \pm 0.04	0.72 \pm 0.04	12 \pm 6
Cerebellum	0.83 \pm 0.07	0.91 \pm 0.07	9 \pm 8
Hindbrain	0.84 \pm 0.07	0.93 \pm 0.06	11 \pm 7

Figure 37: The effect of Stresscopin (SCP) (0.1 μ g) on LCGU (measured by autoradiography) from baseline (mean \pm SEM) (Saline n=12, 0.1 μ g SCP n=11).

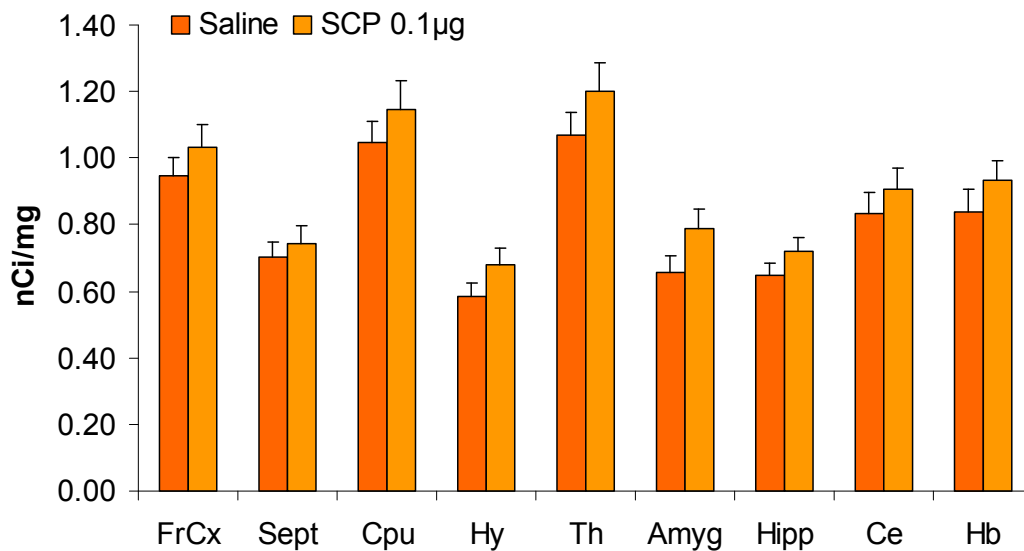
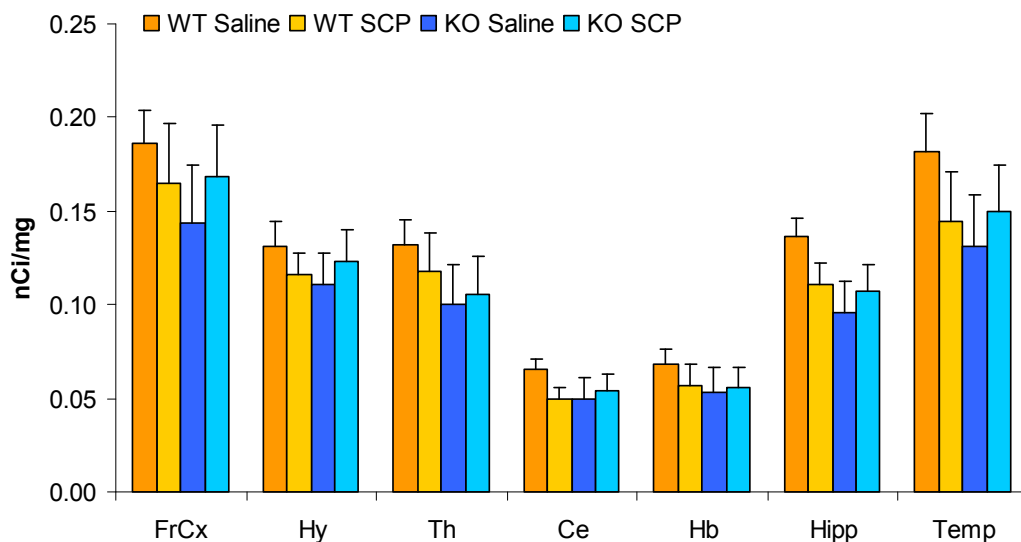


Table 14: Changes in LCGU from baseline (mean \pm SEM) after ICV administration of 0.1 μ g SCP in mice lacking the CRF₁ receptor (CRF1KO) compared to wild-type controls (WT) (WT+Saline n=6, WT+SCP n=6, KO+Saline n=5, KO+SCP n=6).

SCP in CRF1KO Region	LCGU (nCi/mg)				% change in LCGU	
	WT+Saline	WT+SCP	KO+Saline	KO+SCP	WT+SCP	KO+SCP
Frontal Cortical	0.19 \pm 0.02	0.16 \pm 0.03	0.14 \pm 0.03	0.17 \pm 0.03	-12 \pm 17	17 \pm 19
Hypothalamus	0.13 \pm 0.01	0.12 \pm 0.01	0.11 \pm 0.02	0.12 \pm 0.02	-11 \pm 9	11 \pm 15
Thalamus	0.13 \pm 0.01	0.12 \pm 0.02	0.10 \pm 0.02	0.11 \pm 0.02	-11 \pm 16	6 \pm 21
Cerebellum	0.07 \pm 0.01	0.05 \pm 0.01	0.05 \pm 0.01	0.05 \pm 0.01	-24 \pm 9	9 \pm 18
Hindbrain	0.07 \pm 0.01	0.06 \pm 0.01	0.05 \pm 0.01	0.06 \pm 0.01	-17 \pm 17	5 \pm 19
Hippocampus	0.14 \pm 0.01	0.11 \pm 0.01	0.10 \pm 0.02	0.11 \pm 0.01	-19 \pm 9	11 \pm 15
Temporal	0.18 \pm 0.02	0.14 \pm 0.03	0.13 \pm 0.03	0.15 \pm 0.03	-20 \pm 14	14 \pm 19

Figure 38: Changes in LCGU from baseline (mean \pm SEM) after ICV administration of 0.1 μ g SCP in mice lacking the CRF₁ receptor (CRF1KO) compared to wild-type controls (WT) (WT+Saline n=6, WT+SCP n=6, KO+Saline n=5, KO+SCP n=6).



Residual plasma 2DG and Blood glucose

The effects of the treatments in the above studies on the control parameters of residual 2DG in the plasma and blood glucose after the LCGU protocol are summarized in Table 15.

Residual 2DG in the plasma was increased by CRF treatment in four of the described studies: CRF treatment alone (0.5 µg, dissection ($p=0.009$) and autoradiography (trend $p=0.052$) studies); CRF treatment combined with R278995 (2 way ANOVA treatment effect, $F_{3,28}=5.018$, $p=0.033$); and CRF treatment alone in the study combining CRF and antisauvagine-30 ($p=0.020$).

R278995 induced an increase in blood glucose independent of CRF treatment (2 way ANOVA treatment effect, $F_{3,28}=19.070$, $p<0.001$). Increased blood glucose was also seen in mice lacking the CRF₁ receptor, independent of treatment in the Stresscopin study (2 way ANOVA treatment effect, $F_{3,19}=11.639$, $p=0.003$), but a similar increased level was not seen in the earlier study using these mutant mice (CRF treatment). When CRF treatment was combined with antisauvagine-30, blood glucose was reduced, at both the 1 and 3 µg dose ($p=0.003$ and 0.010 , respectively).

Table 15: The effects of CRF system manipulations, alone and in combination, on parameters measured in the blood: residual plasma 2DG, blood glucose and plasma corticosterone. Data are shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, (*)trend $p < 0.06$. ^a dissection study, ^b autoradiography study, NM not measured.

LCGU study	Treatment	Residual plasma 2DG (nCi/ml)	Blood glucose (mg/dl)	Plasma corticosterone (ng/ml)
CRF ^a	Saline	84 \pm 8	163 \pm 9	117 \pm 14
	CRF 0.1 μ g	84 \pm 9	160 \pm 10	336 \pm 15 **
	CRF 0.5 μ g	144 \pm 25 **	159 \pm 11	331 \pm 14 ***
	CRF 1 μ g	108 \pm 11	163 \pm 16	356 \pm 43 **
CRF ^b	Saline	162 \pm 12	NM	172 \pm 20
	CRF 0.5 μ g	248 \pm 28 (*)	NM	358 \pm 11 ***
Urocortin 1	Saline	53 \pm 5	135 \pm 6	161 \pm 54
	UCN1 1 μ g	66 \pm 9	133 \pm 8	347 \pm 38 **
	UCN1 3 μ g	60 \pm 8	118 \pm 9	341 \pm 71 **
CRF in CRF1KO	WT+Saline	169 \pm 15	154 \pm 7	218 \pm 23
	WT+CRF	188 \pm 11	166 \pm 16	305 \pm 32
	KO+Saline	202 \pm 13	176 \pm 10	5 \pm 2
	KO+CRF	225 \pm 27	183 \pm 22	7 \pm 3
R278995/CRF	Ctrl+Saline	148 \pm 38	131 \pm 6	107 \pm 12
	Ctrl+CRF	203 \pm 38	121 \pm 6	344 \pm 12
	R+Saline	109 \pm 8	168 \pm 8	103 \pm 12
	R+CRF	199 \pm 35	187 \pm 20	332 \pm 15
antisauvagine-30/CRF	Saline	131 \pm 9	174 \pm 10	121 \pm 11
	CRF 1 μ g	183 \pm 18 *	142 \pm 13	325 \pm 17 ***
	CRF+1 μ g aSVG	151 \pm 13	124 \pm 10 *	315 \pm 13 ***
	CRF+3 μ g aSVG	133 \pm 16	133 \pm 9 *	304 \pm 9 ***
antisauvagine-30	Saline	71 \pm 5	128 \pm 8	102 \pm 9
	aSVG 1 μ g	70 \pm 8	130 \pm 6	95 \pm 9
	aSVG 5 μ g	71 \pm 7	126 \pm 7	86 \pm 8
Urocortin 2	Saline	151 \pm 13	125 \pm 8	164 \pm 11
	UCN2 1 μ g	184 \pm 13	123 \pm 6	265 \pm 14 **
	UCN2 10 μ g	188 \pm 21	127 \pm 5	250 \pm 6 ***
Stresscopin ^a	Saline	223 \pm 46	168 \pm 12	117 \pm 13
	SCP 0.1 μ g	183 \pm 23	144 \pm 8	161 \pm 11 *
	SCP 1 μ g	250 \pm 31	138 \pm 7	274 \pm 6 ***
	SCP 5 μ g	246 \pm 56	159 \pm 6	306 \pm 11 ***
Stresscopin ^b	Saline	173 \pm 7	NM	NM
	SCP 0.1 μ g	185 \pm 20	NM	NM
SCP in CRF1KO	WT+Saline	164 \pm 19	150 \pm 8	287 \pm 14
	WT+SCP	122 \pm 20	134 \pm 13	281 \pm 39
	KO+Saline	142 \pm 20	176 \pm 8	10 \pm 5
	KO+SCP	144 \pm 8	187 \pm 14	13 \pm 5

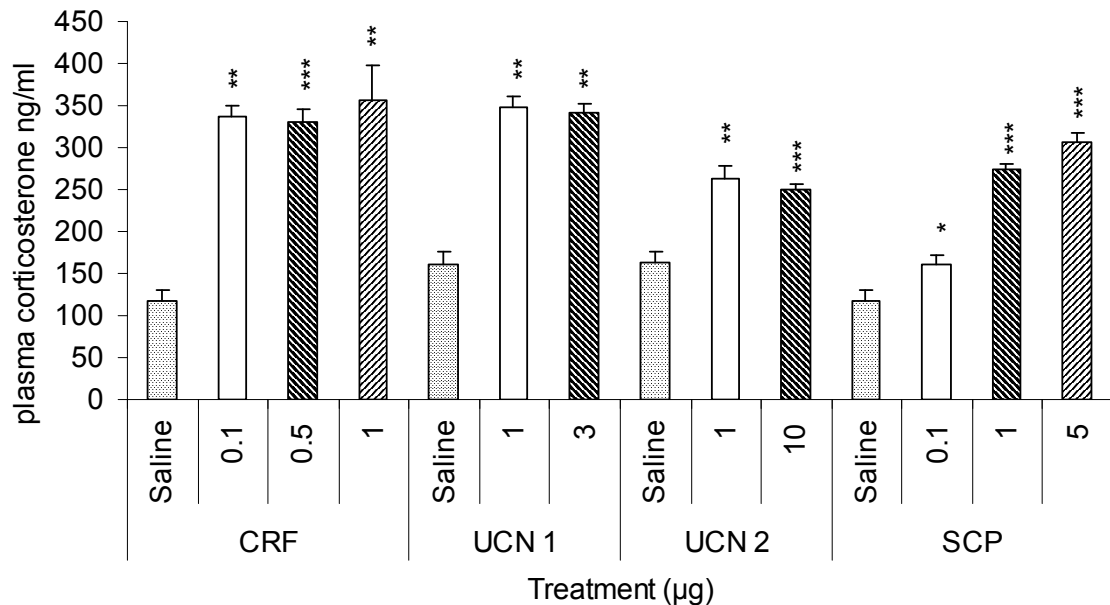
Effects on the HPA axis

Unexpectedly, plasma corticosterone levels were increased by all four of the endogenous CRF-related peptides (Table 15; Figure 39). In the dissection study, CRF induced a greater than twofold increase in plasma corticosterone at all doses (all $p < 0.01$). In the autoradiography study, a similar effect was seen ($p < 0.001$). UCN1 also increased plasma corticosterone at both doses (both $p < 0.01$). The effect of UCN2 on plasma corticosterone was less pronounced, but still highly significant at both doses tested (both $p < 0.01$). SCP induced a dose-dependent increase in plasma corticosterone, with greater than two-fold increases after treatment with 1 and 5 μg (0.1 μg $p < 0.05$, 1 and 5 μg $p < 0.01$).

In CRF1KO mice, the increase in plasma corticosterone after 0.5 μg CRF treatment in wild-type mice ($p < 0.05$) was abolished, and plasma corticosterone in saline-treated mice was greatly diminished (2-way ANOVA genotype effect, $F_{1,27}=4.511$, $p=0.043$). This genotype effect was mimicked in the second study with these mutant mice, although in this study 0.1 μg SCP did not affect plasma corticosterone in either wild-type or mutant mice.

Neither the CRF₁-selective antagonist R278995 or the CRF₂-selective antagonist antisauvagine-30 had any effect on the CRF-induced increases in plasma corticosterone. 1 μg CRF treatment increased plasma corticosterone in both saline- and R278995-pretreated animals (2-way ANOVA treatment effect $F_{3,28}=328.174$, $p < 0.001$). 1 μg CRF increased plasma corticosterone in both saline- and antisauvagine-30-pretreated animals ($p < 0.001$ for all treatment groups).

Figure 39: The effects of the endogenous CRF-related peptides CRF, UCN1, UCN2 and SCP on plasma corticosterone. Data are means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by Kruskal-Wallis followed by Mann-Whitney U tests. (n=5-11 mice per group).



Discussion

The present study aimed to investigate the hypothesis that CRF and the Urocortins induce differing patterns of local cerebral glucose utilisation (LCGU), an indicator of neuronal activity, after central administration, and to further investigate the role of the CRF₁ and CRF₂ receptors in these responses. This was achieved using CRF and the CRF-related peptides, selective antagonists and mutant mice as pharmacological tools. The non-specific CRF₁/CRF₂ agonists CRF and Urocortin 1 both induced increases in LCGU after ICV administration, although with differing LCGU patterns, and the CRF-induced increase in LCGU could be antagonised by the CRF₂-selective antagonist antisauvagine-30, but not the CRF₁-selective antagonist R278995. However, the CRF₂-selective agonists Urocortin 2 and Stresscopin had no significant effect on LCGU.

An increase in LCGU after central administration of CRF is in direct agreement with previous studies in developing and adult rats (Sharkey et al.,

1989; Dube et al., 2000; Freo et al., 2005), although the present studies have identified significant effects in multiple regions at a far lower dose. At doses in a similar range to ours (0.1-1 μ g), Freo et al. (2005) found LCGU increases only in prefrontal cortex, cerebellum and some brainstem regions. At a higher dose of 10 μ g, more widespread increases in LCGU were found, including hippocampus, amygdala, subthalamic nucleus, hypothalamus and in further brainstem regions. At a dose of approximately 3 μ g CRF in developing rats, Dubé et al. (2000) reported increases in LCGU in frontal cortical, amygdala, hippocampal, thalamic and brainstem regions, but not the hypothalamus. At the very high dose of approximately 25 μ g CRF, Sharkey et al. (1989) found increased LCGU in frontal cortical, thalamic, hypothalamic (in particular in the lateral hypothalamus, a region with CRF projections to the thalamus), cerebellum and a number of mid- and hindbrain regions. The widespread effect of CRF on LCGU revealed in the present studies may reflect a drawback of the modifications to the technique used here. A number of the regions studied are components of the limbic system, and indeed all regions studied express CRF or related peptides and CRF receptors. Thus it could be expected that a measurement such as LCGU, taken at the end of a period of receptor stimulation, would show effects in many regions, as the technique cannot delineate whether regions are activated in a particular order.

The complete antagonism of CRF-induced increases in LCGU by the CRF₂-selective antagonist antisauvagine-30 clearly implicates the CRF₂ receptor in this LCGU response. The failure of the CRF₁-selective antagonist R278995 to attenuate the CRF-induced increases in LCGU, and the continued LCGU response to CRF in mice lacking a functional CRF₁ receptor, support this conclusion and further suggest that any involvement of CRF₁ in the LCGU response to CRF is a small one. However, the failure of the CRF₂-selective agonists Urocortin 2 and Stresscopin to alter LCGU contradicts a hypothesised primary role of CRF₂ in LCGU increases.

The use of a stressful technique for the ICV injection (see Chapter 2, Study 5) of CRF and the Urocortins may have adversely affected the LCGU results obtained, although a clear effect of the ICV injection technique alone on LCGU was not seen (Chapter 2, Study 5). It is possible that the effects of CRF on

LCGU seen in the present studies represent a potentiation of stress-induced effects on LCGU brought about by the freehand ICV injection procedure. However, as stress has been reported to have clear effects on LCGU in the literature (Soncrant et al., 1988; Xing et al., 1990; Duncan et al., 1993; Frietsch et al., 2000; Takamatsu et al., 2003a, 2003b; and considered later in this thesis), the lack of a clearly detectable effect of the ICV procedure on LCGU found in Chapter 2 suggests that the degree of stress induced by the procedure is not sufficient to significantly alter LCGU. Despite these considerations, the clear effect of antisauvagine-30 on CRF-induced changes in LCGU continues to implicate CRF₂ receptors in the LCGU response to CRF, in contrast to the effects of Urocortin 2 and Stresscopin. The extent of any influence of the ICV procedure used could be investigated through the replication of the key elements of the present data using pre-implanted cannulae for ICV injection, which under well-controlled conditions constitute a minimal stressor for the animal.

Ligand-specific receptor populations within overall CRF₁ or CRF₂ populations may explain the lack of effect of Urocortin 2 and Stresscopin on LCGU despite the evidence that CRF-induced changes in LCGU are CRF₂-mediated. Indeed a new concept of ligand-dependent differential regulation of receptor-coupled effector pathways specifically includes agonist-specific signaling (Urban et al., 2007). Such functional selectivity has been suggested for a range of neurotransmitter-receptor systems, including in the serotonergic, opioid, dopaminergic, vasopressin and adrenergic systems (Urban et al., 2007). Urocortin 1 and CRF have been shown to regulate G-protein receptor kinase activity differently in human retinoblastoma Y79 cells (Dautzenberg and Hauger, 2002). Such a difference may account for the differing effect size of Urocortin 1 on LCGU compared to CRF, and allows speculation that further differences in receptor activation may be present for Urocortin 2 and Stresscopin. Ligand binding in CRF₁ requires ligand-receptor interaction at four points: between the ligand C-terminal and the receptor N-terminal, and between the ligand N-terminal and the receptor juxtamembrane (J)-domain (Perrin and Vale, 1999; Beyermann et al., 2000; Hoare et al., 2004). Whether a similar dual domain model exists for CRF₂ has not yet been clarified (Hoare et al., 2005). The J-domain in CRF₁ has been linked to coupling with either G_s or G_i through ligand-induced conformational changes (Berger et al., 2006). G-protein interaction with the CRF₂

J-domain has also been reported to affect the affinity of Urocortin 2 and Stresscopin (Hoare et al., 2005), although the effects on CRF or Urocortin 1 binding were not included in this study for comparison. Therefore it is possible that different interaction with the CRF₂ J-domain for the different CRF-related peptides studied presently may explain the discrepancies between the effects on LCGU via CRF₂.

Further studies are necessary to determine the rate at which Urocortin 2 and Stresscopin diffuse from the ventricles after ICV administration, as while it has been shown that CRF and Urocortin 1 access cognate receptors after ICV injection (Bittencourt & Sawchenko, 2000), it is possible that Urocortin 2 and Stresscopin do not diffuse as readily. Indeed, delayed permeation of Urocortin 2 from the ventricles to sites of CRF₂ expression has been proposed (Bale & Vale, 2004) as a mechanism explaining the delayed effects of this peptide on anxiety-related behaviour (Valdez et al., 2002). Such time-dependent effects may also be present in the LCGU response to Urocortin 2 or Stresscopin, an aspect that could also be studied further, although the clear effects of Urocortin 2 or Stresscopin on the HPA axis indicate that these peptides have reached CRF₂ receptors in the hypothalamic paraventricular nucleus (PVN) or regions projecting to the PVN.

Ligand-specific receptor activation may also explain the differences in the LCGU response to Urocortin 1 compared to CRF, despite Urocortin 1's higher affinity for both CRF₁ and CRF₂ compared to CRF. A subpopulation of the brain's CRF receptors may be selective for Urocortin 1, and this subpopulation may correspond to the endogenous distribution of Urocortin 1. As this endogenous distribution is substantially smaller than that of CRF, the effect of exogenous Urocortin 1 applied to the brain may also be expected to be less widespread. It has also been suggested that Urocortin 1 may act to displace CRF from the CRF-binding-protein (CRF-BP) (Behan et al., 1996). Such a mechanism could also be involved in Urocortin 1-induced increases in LCGU.

Chen et al (2005) have identified a soluble version of the CRF₂ receptor in the brain, expressed in areas that also express CRF₁. This variant of the CRF₂ receptor binds Urocortin 2 and 3, but also CRF and Urocortin 1 with high affinity,

and the authors propose that it may modulate ligand activity by either competing with the full-length, membrane-bound receptors, by presenting the ligand or by prolonging its action. Furthermore, the existence of alternative receptors with distributions different to those of the membrane-bound receptors may account for some of the complex and often varied effects of CRF ligand administration.

The CRF₁ receptor has consistently been implicated in the anxiogenic effects associated with CRF through a combination of several antagonist and mutant mouse studies (comprehensively reviewed in Steckler, 2005 and Takahashi, 2001). Thus, it is possible that the clear involvement of CRF₂ in the LCGU response could reflect a specific CRF₂-mediated glucose metabolism pathway, linking with reports of peripheral glucose homeostasis mechanisms involving CRF₂ (Chen et al., 2006), rather than the LCGU responses measured here reflecting a state during (e.g.) anxiety-related behaviours. However, studies have shown that CRF₂ may also be involved in anxiety-related behaviour that can also be CRF₁-mediated (Risbrough et al., 2003; Risbrough et al., 2004), and have further shown that stimulation of CRF₂ in the lateral septum affects anxiety-related behaviour (Todorovic et al., 2007), although CRF₂ stimulation in this region can either increase or decrease anxiety-related behaviour depending on the basal stress-state of the animal (Henry et al., 2006). Therefore an alternative explanation is that some or all of the effects of CRF on LCGU in the present studies propagate from CRF₂-mediated effects in the lateral septum, which would explain their antagonism by antisauvagine-30.

In those studies examining the effects of CRF on LCGU, CRF consistently increased LCGU in multiple brain regions. However, while LCGU was increased in the cerebellum and hippocampus in all CRF studies, effects in the frontal cortex, hypothalamus and thalamus were less robust. For example, while the effect of CRF on LCGU in dissected thalamus was large, in the same region measured using autoradiography (although actual nCi/mg values differed due to the varying specific activity of the two radioactive isotope forms of 2DG used, ³H and ¹⁴C) no significant effect was found. This may simply be dose related, as at the 0.5 µg dose used in the autoradiography study, only a trend was seen in dissected thalamus. However, in the later study examining the effects of R278995 on CRF-induced LCGU changes, no significant effect was found in the

dissected thalamus even at the 1 μ g dose used. This discrepancy may be due to increased variation between individual responses in the latter study. However, increased experimental variation may also have been introduced by the stressful nature of the freehand ICV injection procedure. While the overall effect of CRF in increasing LCGU is supported by studies using pre-implanted, less-stressful, cannulae (Sharkey et al., 1989; Dube et al., 2000; Freo et al., 2005), the robustness of the CRF effect in particular regions may have been reduced through the freehand ICV procedure.

CRF increased hypothalamic LCGU in three out of five studies. While the lack of a robust effect in this region may again be due to the techniques used, a CRF-induced increase in hypothalamic LCGU is consistent with activation of the HPA axis, confirmed by the large increase in plasma corticosterone. However, hypothalamic LCGU was not significantly increased in Urocortin 1-, Urocortin 2- or Stresscopin-treated animals, despite increased plasma corticosterone, also suggesting HPA axis activation. Thus, altered hypothalamic LCGU may be unrelated to activation of the HPA axis, although effects purely in the paraventricular nucleus (PVN; the source of CRF signaling to the pituitary) could be masked by effects in other hypothalamic regions in the dissected sample. Indeed, CRF₁-mediated neuronal activation and CRF₂-mediated neuronal suppression has also been reported in the ventromedial hypothalamus (McCrimmon et al., 2006), illustrating the possibility that the effects measured in complete hypothalamus do not necessarily represent the PVN.

As described earlier (Introduction) central administration of CRF has also been reported to increase c-Fos expression in widespread regions throughout the rodent brain (Bittencourt and Sawchenko, 2000), and matching the regions exhibiting increased LCGU after CRF administration in the present studies. Notably, the effects of central Urocortin 1 administration on c-Fos expression closely resembled those to CRF (Bittencourt and Sawchenko, 2000), though to a slightly reduced degree at a matching dose. This is in contrast with the effects on LCGU seen in the present studies, in which the effects of Urocortin 1 on LCGU were much less widespread, despite testing at a higher dose.

LCGU increases after ICV CRF may correspond to behavioural activation, which is a reported effect of ICV CRF when the animal is in a familiar environment (Dunn and Berridge, 1990), as was the case here, where the animals were kept in their home cage during the experimental protocol. As a region implicated in the motor control of movement, the increased LCGU seen in the cerebellum could support this hypothesis. LCGU in the cerebellum could be directly mediated by CRF₂, as evidence for expression of this CRF receptor subtype in this region has been provided (Bishop et al., 2000; Gounko et al., 2006), although contradictory findings reporting an absence of CRF₂ in the cerebellum have also been published (Van Pett et al., 2000). CRF-induced LCGU increases may also reflect increased arousal, also associated with central administration of CRF (Warnock et al., 2006; Sauvage and Steckler, 2001), and with a number of brain regions, including the laterodorsal and pendunculo pontine tegmental nuclei, dorsal raphe and locus coeruleus, regions included in the dissected hindbrain region, which showed up to 40% increase (though reaching only trend-like significance in the autoradiography study, although this discrepancy may reflect technical issues as discussed above) in LCGU after CRF administration. This highlights one of the discussed drawbacks of measuring LCGU in dissected brain regions as used presently, as the dissection of large brain regions can lead to the amalgamation of small, but interesting, regions.

Also of note, the regions displaying altered LCGU in the present study after CRF-related peptide treatment do not entirely correspond to regions predominantly expressing either CRF₁ or CRF₂ receptors. For example, CRF induced an increase in thalamic LCGU in most studies, mediated by CRF₂ receptors, despite no reported expression of these receptors in the thalamus (reviewed in Warnock et al., 2006). This would suggest changes in LCGU downstream of CRF-related peptide-induced effects at CRF receptors in other brain regions. Projections from regions directly activated by CRF₂ may propagate CRF's effects to other regions, and interactions with other neurotransmitter systems may also lead to effects in regions not directly stimulated. Indeed, there are numerous CRF projections to the thalamus, originating from regions including the central amygdala and lateral hypothalamus (reviewed in Warnock et al., 2006). As a site of CRF₂ expression, LCGU in the hippocampus may be

directly influenced by CRF administration. In addition, as discussed above, the cerebellum may also be a site of direct CRF₂-mediated influence. Although the expression of CRF₂ in the cerebellum is contested, as discussed above, in the absence of CRF₂ expression and as a region displaying one of the most robust CRF-induced LCGU increases, the cerebellum becomes a likely candidate for effects downstream of effects in other regions.

In addition to the putative links with behavioural activation and motor activity through CRF-induced increased LCGU in the cerebellum, a number of the other regions displaying altered LCGU after CRF administration in the present studies have been implicated in stress-related function.

Increased LCGU in the dissected hindbrain region may include altered LCGU in regions such as the LC, and PPTg, both regions linked to arousal and attention (Carli et al., 1983; Steckler et al., 1994; Usher et al., 1999; Inglis et al., 2001), and sites through which CRF may interact with noradrenaline and acetylcholine to modulate these processes (reviewed in Warnock et al., 2006). Furthermore, the cholinergic PPTg and LDTg project to the cerebellum, raphe nuclei, thalamus, hypothalamus, basal forebrain and medial prefrontal cortex (Satoh and Fibiger, 1986; Butcher, 1992; Steckler et al., 1994), providing further possibilities of CRF-induced secondary effects on LCGU, through interaction with other neurotransmitter systems. Furthermore, a feed-forward loop originating in the LC and projecting through the noradrenergic system to the PVN, BNST and CeA has been suggested, with a hypothesised involvement in central activation during stress-related situations (Koob, 1999). In turn, the PVN, BNST and CeA have been implicated as a source of CRF innervation to the ventral tegmental area (Rodaros et al., 2007), where CRF release has been linked to stress-activation of appetitive behaviour (Wang et al., 2005). Also included in the dissected hindbrain regions, are the ventrolateral medulla, dorsomedial medulla/NTS and ventrolateral pons which have been implicated in several integrative functions, including the relaying of primary viscerosensory information and in sympathetic activation (Ingram, 2005).

CRF also induced a trend towards increased LCGU in the amygdala when this region was examined more specifically by autoradiography. The CeA and

MeA are involved in the processing and relaying of stress signals to other limbic and brainstem areas (Davis et al., 1994), and the CeA has also been linked to the autonomic effects of CRF through activation of the sympathetic nervous system (Dunn and Berridge, 1990; Gray, 1993; Gray and Bingaman, 1996). The BLA plays an important role in the integration of inputs from various cortical and thalamic sites and may also play an important role in associative learning which underlies fear conditioning and anxiety (Ingram, 2005).

The hippocampus, which exhibited clear, robust, increases in LCGU in response to CRF, has a role in episodic memory, particularly for the emotional context of memories, and plays a role in regulating behavioural responses to threatening environmental contexts (Phillips and LeDoux, 1992). As a part of the septo-hippocampal system, the hippocampus may be also involved in anxiety through a behavioural inhibition system (Gray, 1983; Gray and McNaughton, 2000). This region is also a likely site of interaction between the CRF and cholinergic systems and their involvement in the modulation of cognitive behaviour (reviewed in Warnock et al., 2006).

In addition to the postulated role of CRF₂ receptors in the septum in the propagation of CRF-induced effects on LCGU, above, the septal area is involved in the regulation of hippocampal function through the septohippocampal pathway, a connection that is important in controlling the termination of stress responses (Ingram, 2005).

The dissected frontal cortical region also contained aspects of the prefrontal cortex. This region may be involved in the integration of both stress-induced activation of the HPA axis and glucocorticoid negative feedback with executive function and cognitive processing (Sullivan and Gratton, 2002b), and is implicated in generating an active coping strategy in response to a stressful stimulus (Giorgi et al., 2003), perhaps setting an emotional tone to information processing (Ingram, 2005).

The effects of the endogenous CRF-related peptides tested on LCGU do not support theories of opposing roles for these receptors in the brain (Skelton et al., 2000; Chen et al., 2003), although care must be taken without direct

translation of effects on LCGU into behaviour. It may also be prudent to examine the effects of Urocortin 2 and Stresscopin on LCGU using an alternative method for ICV injection, as the freehand technique used presently reflects one of the largest drawbacks and potential confounds in the present studies. When one considers the somewhat unclear literature on the role of CRF₂ in e.g. anxiety-related behaviour (Takahashi, 2001; Takahashi, 2002), it is possible that LCGU simply represents one physiological measurement which can be altered by CRF-related peptides. This particular parameter may not be directly translatable to behavioural effects, despite its demonstrated ties to neuronal activation (see Introduction), and may be affected through unrelated mechanisms and different signaling pathways. Indeed, increased HPA axis activity, as demonstrated by increased plasma corticosterone, after stimulation of either CRF receptor, despite otherwise unmatching central effects on LCGU, suggests an uncoupling of HPA axis and central responses. As 2DG itself only increased plasma corticosterone at doses hundreds-fold greater than that used to measure LCGU (see Chapter 2), the corticosterone responses to CRF and the Urocortins can not be attributed to this aspect of the protocol.

CRF has been reported to increase blood glucose in the literature (Brown et al., 1982a), although the dose used in this study (>25 µg) was much larger than those necessary to exert behavioural effects (<1 µg). Sufficient changes in blood glucose could violate the assumptions of the LCGU model, leading to unreliable conclusions. No effect on blood glucose at the end of the LCGU protocol after CRF treatment was found in the present studies, suggesting that the assumptions were fulfilled. However, the small increases in residual plasma 2DG seen after CRF treatment may indicate transient changes in blood glucose during the LCGU protocol, not detected by end point measurements. Increased blood glucose would compete with 2DG as an energy source for neurons, thus effectively reducing measured LCGU as the effective dose of 2DG is reduced. Therefore, the detection of increased LCGU after CRF treatment in the present studies, despite this minor evidence of changing blood glucose (including the state increase in blood glucose found after R278995 treatment), further indicates the robustness of the overall LCGU response to CRF (despite less robustness in the LCGU effects in particular regions as discussed above). Furthermore, the 45 minute duration of the LCGU protocol was chosen to allow nearest-to-full

metabolism of the injected dose of 2DG, meaning that a large increase in competition between endogenous glucose and 2DG would be necessary to influence measurements to a physiologically significant level. Assuming an approximate injected dose of 18 μCi 2DG (at the 600 $\mu\text{Ci/kg}$ dose used), residual 2DG levels of even 200 nCi/ml (compare to Table 15) represent less than 2 % of the initial dose.

Reduced blood glucose in combined CRF and antisauvagine-30 treated groups agrees with the literature for the periphery, in that peripheral Urocortin 2 and the CRF_2 receptor in skeletal muscle are reported to be involved in the negative regulation of glucose uptake (Chen et al., 2006). Antisauvagine-30 increased skeletal muscle glucose uptake, leading to reduced blood glucose (Chen et al., 2006). However, it should be noted that this study administered Urocortin 2 or antisauvagine-30 peripherally, compared to the ICV route used in the present studies. Centrally, Urocortin 3 has been reported to increase sympathetic activity in a similar fashion to CRF (Brown et al., 1982b; Dunn and Berridge, 1990), including increasing blood glucose (Jamieson et al., 2006). Therefore, in the present studies, reduced blood glucose in antisauvagine-30 treated mice may support a mild, CRF_2 -mediated, effect on sympathetic activity.

Increased plasma corticosterone levels after Urocortin 2 or Stresscopin administration suggests that CRF_2 plays a role in HPA axis activation. In support of this, de Groote et al. (2005) found increased levels of corticosterone in the rat hippocampus after not only CRF and Urocortin 1 but also after Urocortin 3 treatment. In contrast, some studies have suggested that CRF_2 does not play a major role in the acute response of the HPA axis to stress. A number of studies have reported that baseline plasma ACTH levels were unaltered by either Urocortin 2, Stresscopin or the selective CRF_2 antagonist antisauvagine-30 (Ruhmann et al., 1998), administered either peripherally (Hsu and Hsueh, 2001) or centrally (Pellemounter et al., 2002; 2004), although antisauvagine-30 slightly attenuated the ACTH response to ICV injection alone (Pellemounter et al., 2002). However, Urocortin 2 mRNA is up-regulated in the parvocellular part of the rat PVN following immobilisation stress and in the magnocellular part following water deprivation, raising the possibility that this highly selective CRF_2 agonist may be involved in stress-induced HPA axis responses (Tanaka et al.,

2003). A greater impairment of the HPA axis response to stress has also been reported in mice lacking both CRF₁ and CRF₂, compared to mice lacking CRF₁ alone (Bale et al., 2002b), further supporting a role for CRF₂ in the HPA axis. Most recently, clear CRF₂-mediated activation of the HPA axis, demonstrated though increased CRF heteronuclear RNA in the PVN and increased plasma ACTH, has been reported after central administration of Urocortin 2 or Urocortin 3 (Stresscopin) (Maruyama et al., 2007), in direct agreement with the HPA axis activation demonstrated by increased plasma corticosterone found in the present studies.

Despite a lack of effect in the present LCGU model, Urocortin 2, as a tool for agonism of CRF₂ receptors, may affect other factors linked to neuronal activation. Indeed, Urocortin 2 has been reported to increase extracellular serotonin in the basolateral amygdala (Amat et al., 2004), and also in the hippocampus (de Groote et al., 2005), a site of reported CRF₂ expression (see Warnock et al., 2006). Furthermore, Urocortin 2 has been reported to increase c-Fos expression in subpopulations of serotonergic neurons within specific subdivisions of the rat dorsal raphe nucleus (Amat et al., 2004; Staub et al., 2005; Staub et al., 2006), a part of the dissected hindbrain region, and reported site of CRF₂ expression. Furthermore, a number of regions contained in the dissected hindbrain regions are sites of Urocortin 1, 2 and 3 expression, suggesting endogenous CRF₂-related activity in this part of the brain. These findings support the hypothesised role of CRF₂ in the mediation of neuronal activation, despite the lack of activity of this ligand in the present model.

However, it has been reported that direct administration of Urocortin 2 into the dorsal raphe inhibited neuronal activity at low doses, while a higher dose of Urocortin 2 showed mixed effects, activating some neurons, while inhibiting others (Pernar et al., 2004). These effects could be blocked with the CRF₂-selective antagonist antisauvagine-30, but not the CRF₁-selective antagonist antalarmin (Webster et al., 1996), indicating that the effects are CRF₂ mediated. A bimodal mechanism for CRF₂-induced neuronal inhibition or activation such as this may mask effects on LCGU, meaning that changes in LCGU in particular regions could be missed.

The present findings add useful information to the body of literature on the function of CRF₁ and CRF₂ receptors, and CRF-related peptides. In conclusion, a role for the CRF₂ receptor in the modulation of central glucose utilisation is postulated, but the similar effects of the CRF-related peptides on plasma corticosterone suggest that the HPA axis response may be uncoupled from the central effects of CRF receptor activation.

Chapter 5 – The Effect of Restraint Stress on LCGU and Comparison to the Effects of CRF

The Effect of Restraint Stress on LCGU and Comparison to The Effects of CRF

Introduction/Aims

As discussed earlier, the CRF system has been implicated in the stress response, both in the context of stress-related behaviour and in the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Thus, in order to compare the effects of direct manipulation of the CRF system on LCGU to those induced by a stressor, it was decided to study the effect of restraint stress on LCGU.

Restraint stress has been reported to activate the HPA axis (Crine et al., 1983; Odio and Maickel, 1985; Hauger et al., 1988; Rowland and Dunn, 1995; Torres et al., 2001; Rivalland et al., 2007), and has also been reported to increase c-fos expression in regions that express components of the CRF system (Funk et al., 2003; Crane et al., 2005; Funk et al., 2006), such as the hypothalamus, amygdala and locus coeruleus, and specifically including CRF-expressing neurons in the paraventricular nucleus of the hypothalamus (PVN) (Rivalland et al., 2007).

Restraint in cold water has been reported to markedly reduce LCGU globally in rats (Xing et al., 1990), and reductions in LCGU in the dorsal hippocampus and anteroventral thalamic nucleus were also seen after another stressor similar to restraint stress, four limb immobilisation (Soncrant et al., 1988), although an increase was seen in the lateral habenula. However, it should be noted that restraint (and other stressors) have been reported to increase blood glucose levels (Odio and Maickel, 1985; Soncrant et al., 1988; Rowland and Dunn, 1995; Brown et al., 1996; Torres et al., 2001). As has been discussed previously, sufficient variations in blood glucose may influence the measurement of LCGU. The measurement of blood glucose as part of the LCGU protocol allows for the control of this variable.

To study the effects of restraint stress on LCGU and the HPA axis, these parameters were first measured after restraint during the LCGU protocol, and the involvement of the CRF₁ receptor assessed using the selective CRF₁ antagonist

R278995 (Chaki et al., 2004). To further investigate the effects of the restraint stress on LCGU, the effects of various durations of restraint were compared.

Methods

Subjects

Male C57BL/6N mice were used as described in the General Methods.

Compounds

The selective CRF₁ antagonist R278995 (Chaki et al., 2004) co-developed by Johnson & Johnson Pharmaceutical Research & Development and Taisho Pharmaceuticals (Japan) was used to assess the involvement of CRF₁ receptors in the LCGU response to restraint. R278995 or solvent (saline plus 10% cyclodextrin and tartaric acid) was injected subcutaneously (SC) at a dose of 40 mg/kg, 1 hour before the injection of 2DG. The formulation of R278995 in solution was carried out by the J&JPRD Internal Pharmacy.

Measurement of local cerebral glucose utilisation (LCGU) and plasma corticosterone

LCGU and plasma corticosterone were measured as described in the General Methods. ³H-2DG was injected immediately before restraint stress.

Restraint Stress

For the restraint stress, mice were removed from their home cage, injected with 2DG then placed in well ventilated stainless steel restraining tubes for between 2 and 45 minutes. Mice receiving restraint stress for less than the full 45 minute duration of the LCGU protocol were returned to their home cage for the remainder of the protocol duration. 45 minutes after 2DG injection, the mice were either removed from their home cages or the restrainers and decapitated in accordance with the LCGU protocol.

Statistical Analysis

Data were analysed as described in the General Methods. Briefly, the influence of the selective CRF₁ antagonist R278995 on LCGU after restraint stress was analysed by 2-way ANOVA, while the LCGU response to varying durations of restraint stress was analysed by the Kruskal-Wallis test, followed by the Mann-Whitney U-test for post-hoc comparisons.

Results

45 minutes of restraint stress significantly reduced LCGU in all regions studied (2-way ANOVA restraint effect $F_{3,26}=7.962-29.44$, all $p<0.01$) (Table 16; Figure 40), and significantly increased plasma corticosterone levels (2-way ANOVA restraint effect $F_{3,26}=343.206$, $p<0.001$), but had no effect on either residual plasma 2DG or blood glucose (Table 17; Figure 41).

The effects of restraint on LCGU and corticosterone were unaffected by pre-treatment with R278995, as were residual plasma 2DG or blood glucose (Table 16, 17; Figure 40, 41).

Table 16: The effect of 45 minutes restraint stress, and pre-treatment with 40 mg/kg R278995 on LCGU (mean \pm SEM) in mice (control-control n=8 , control-restraint n=8 , R278995-control n=6 , R278995-restraint n=8)

R278995/Restraint Region	LCGU (nCi/mg)			
	Saline+Ctrl	Saline+Restraint	R+Ctrl	R+Restraint
Frontal Cortical	0.36 \pm 0.03	0.28 \pm 0.02	0.32 \pm 0.02	0.25 \pm 0.01
Hypothalamus	0.27 \pm 0.02	0.22 \pm 0.02	0.27 \pm 0.02	0.22 \pm 0.01
Thalamus	0.33 \pm 0.03	0.22 \pm 0.02	0.30 \pm 0.02	0.21 \pm 0.01
Cerebellum	0.22 \pm 0.02	0.15 \pm 0.01	0.20 \pm 0.01	0.13 \pm 0.01
Hindbrain	0.15 \pm 0.01	0.11 \pm 0.01	0.14 \pm 0.01	0.12 \pm 0.01
Hippocampus	0.26 \pm 0.02	0.20 \pm 0.02	0.26 \pm 0.02	0.20 \pm 0.01
Temporal	0.34 \pm 0.02	0.25 \pm 0.02	0.31 \pm 0.02	0.26 \pm 0.02

R278995/Restraint Region	% change in LCGU		
	Saline+Restraint	R+Ctrl	R+Restraint
Frontal Cortical	-24 \pm 5	-12 \pm 5	-30 \pm 2.2
Hypothalamus	-18 \pm 7	-2 \pm 8	-20 \pm 2.6
Thalamus	-32 \pm 7	-10 \pm 8	-35 \pm 2.7
Cerebellum	-33 \pm 4	-10 \pm 7	-42 \pm 4.8
Hindbrain	-29 \pm 5	-7 \pm 9	-25 \pm 4.5
Hippocampus	-23 \pm 6	-2 \pm 6	-24 \pm 3
Temporal	-36 \pm 11	-10 \pm 5	-26 \pm 5.1

Figure 40: The effect of 45 minutes restraint stress, and pre-treatment with 40 mg/kg R278995 on LCGU in mice (mean \pm SEM) (control-control n=8 , control-restraint n=8 , R278995-control n=6 , R278995-restraint n=8) (Region abbreviations as in previous Figures)

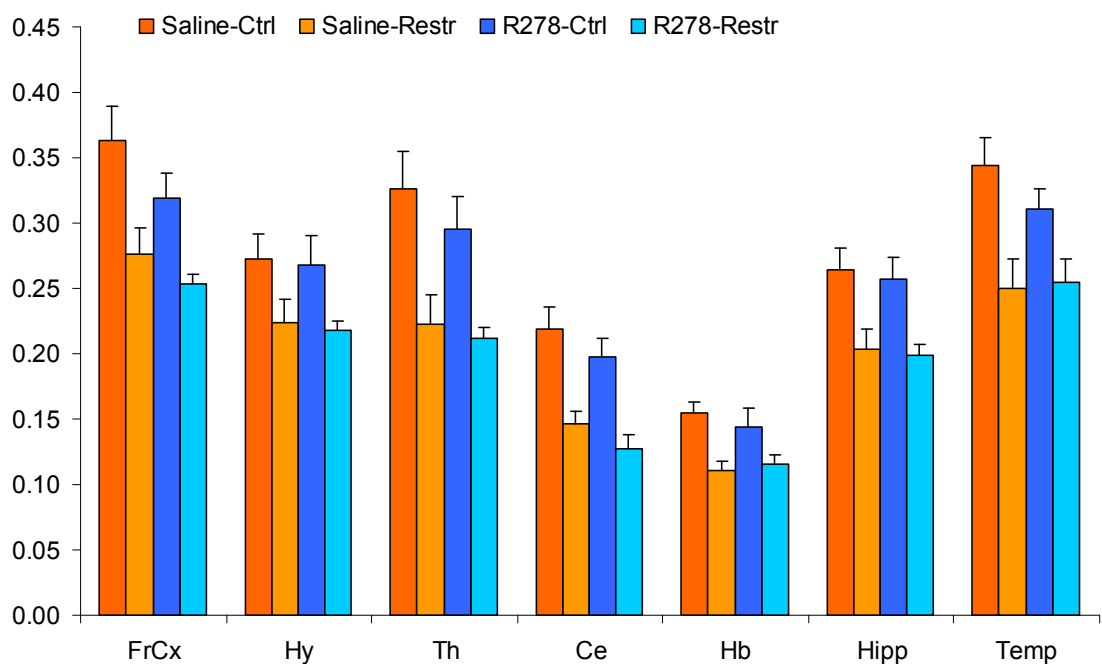
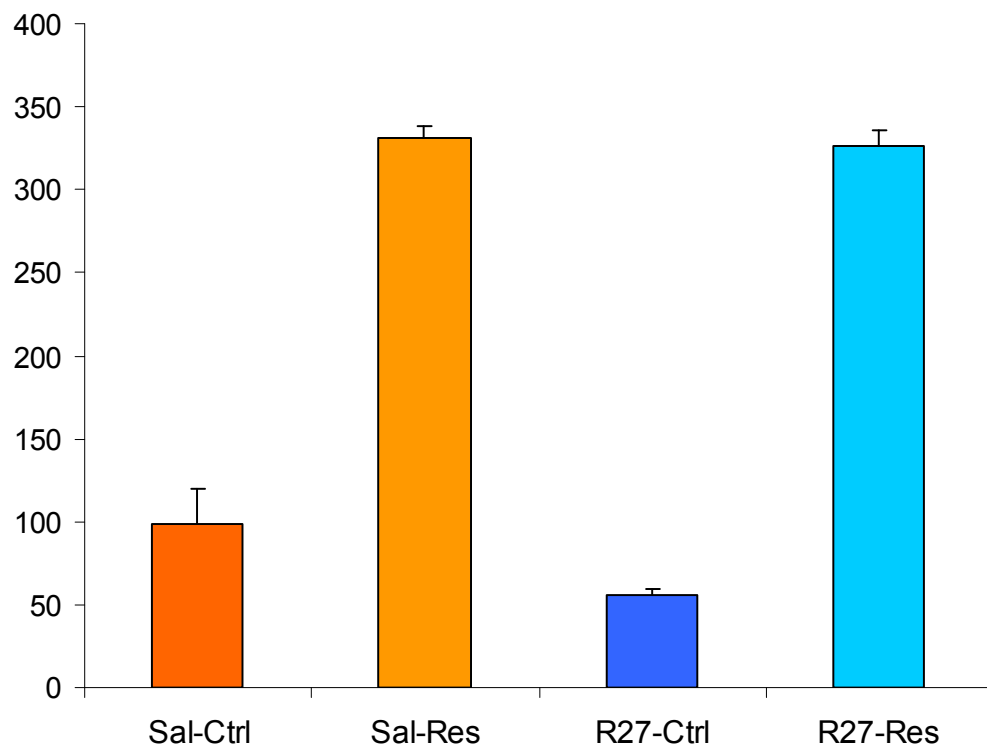


Table 17: The effect of 45 minutes restraint stress, and pre-treatment with 40 mg/kg R278995 on residual plasma 2DG, blood glucose and plasma corticosterone in mice (mean \pm SEM) (control-control n=8 , control-restraint n=8 , R278995-control n=6 , R278995-restraint n=8)

LCGU study	Treatment	Residual plasma 2DG (nCi/ml)	Blood glucose (mg/dl)	Plasma corticosterone (ng/ml)
R278995/ Restraint	Saline+Ctrl	39 \pm 6	135 \pm 8	98 \pm 22
	Saline+Restraint	36 \pm 6	156 \pm 16	331 \pm 8
	R278+Ctrl	37 \pm 8	157 \pm 12	55 \pm 4
	R278+Restraint	38 \pm 7	176 \pm 23	326 \pm 10

Figure 41: The effect of 45 minutes restraint stress, and pre-treatment with 40 mg/kg R278995 on plasma corticosterone in mice (mean \pm SEM) (control-control n=8 , control-restraint n=8 , R278995-control n=6 , R278995-restraint n=8) (key: Sal – saline; Ctrl – control; Res – restraint; R27 – R278995)



When the effect of varying restraint time on LCGU was measured, significant reductions in LCGU were only seen after 45 minutes of restraint in the frontal cortical, thalamic, hippocampal and temporal dissected regions ($p < 0.05$) (Table 18; Figure 42).

No changes in residual plasma 2DG or blood glucose were found after any duration of restraint stress studied (Table 19). Plasma corticosterone levels were increased in a restraint-duration dependent manner (Table 19).

Table 18: The effect of different durations of restraint stress on LCGU (mean \pm SEM) in mice (group sizes $n=8-10$) (* $p < 0.05$)

Restraint Stress Region	Control	2 min	LCGU (nCi/mg)				
			5min	10 min	20 min	45 min	
Frontal Cortical	0.27 \pm 0.02	0.24 \pm 0.03	0.22 \pm 0.01	0.30 \pm 0.02	0.24 \pm 0.01	0.21 \pm 0.02	*
Hypothalamus	0.20 \pm 0.01	0.19 \pm 0.02	0.18 \pm 0.01	0.22 \pm 0.01	0.20 \pm 0.02	0.18 \pm 0.01	
Thalamus	0.22 \pm 0.02	0.19 \pm 0.02	0.18 \pm 0.02	0.24 \pm 0.02	0.20 \pm 0.02	0.15 \pm 0.01	*
Cerebellum	0.13 \pm 0.01	0.11 \pm 0.02	0.12 \pm 0.02	0.13 \pm 0.02	0.10 \pm 0.01	0.09 \pm 0.01	
Hindbrain	0.10 \pm 0.01	0.08 \pm 0.01	0.08 \pm 0.02	0.09 \pm 0.01	0.07 \pm 0.01	0.06 \pm 0.01	
Hippocampus	0.15 \pm 0.01	0.13 \pm 0.01	0.13 \pm 0.01	0.16 \pm 0.00	0.14 \pm 0.01	0.12 \pm 0.01	*
Temporal	0.18 \pm 0.01	0.15 \pm 0.01	0.15 \pm 0.01	0.25 \pm 0.06	0.16 \pm 0.01	0.15 \pm 0.01	*

Restraint Stress Region	2 min	5min	% change in LCGU		
			10 min	20 min	45 min
Frontal Cortical	-12 \pm 10	-18 \pm 5	12 \pm 7	-12 \pm 4	-22 \pm 6
Hypothalamus	-8 \pm 11	-13 \pm 7	11 \pm 6	-2 \pm 9	-13 \pm 5
Thalamus	-14 \pm 11	-19 \pm 8	6 \pm 9	-11 \pm 8	-32 \pm 5
Cerebellum	-18 \pm 13	-13 \pm 12	-6 \pm 13	-23 \pm 10	-30 \pm 7
Hindbrain	-20 \pm 12	-13 \pm 16	-2 \pm 15	-22 \pm 11	-33 \pm 10
Hippocampus	-11 \pm 10	-16 \pm 6	4 \pm 3	-8 \pm 6	-19 \pm 4
Temporal	-16 \pm 8	-16 \pm 6	42 \pm 32	-9 \pm 6	-17 \pm 6

Figure 42: The effect of different durations of restraint stress on LCGU (mean \pm SEM) in mice (group sizes n=8-10) (*p<0.05)

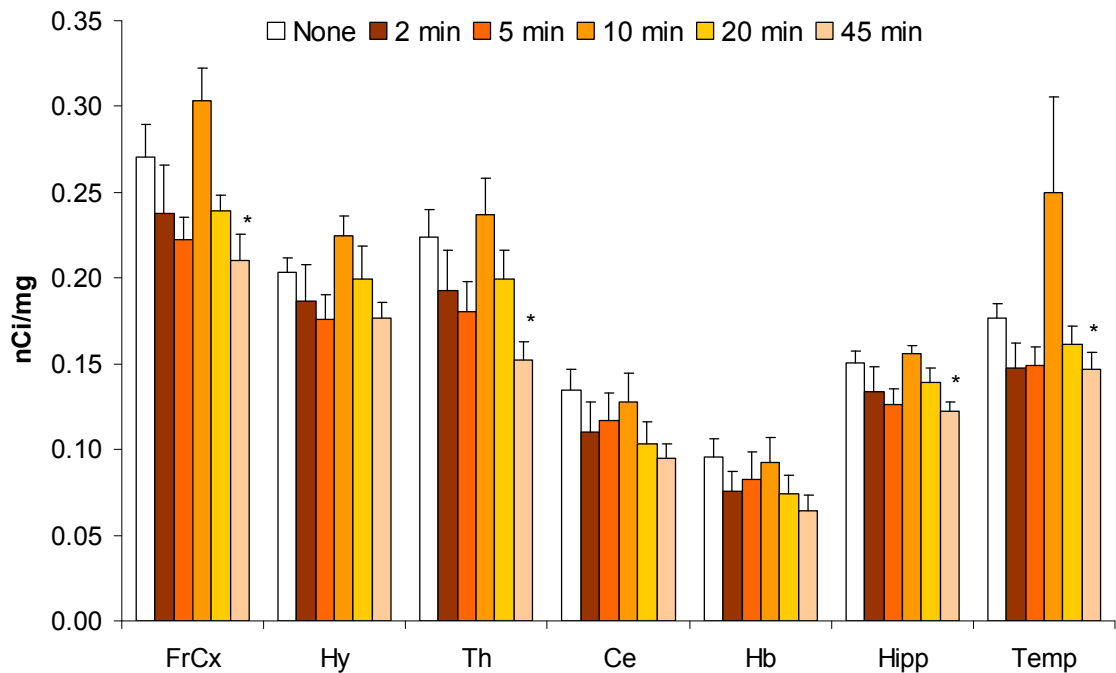


Table 19: The effect of different durations of restraint stress on residual plasma 2DG, blood glucose and plasma corticosterone in mice (mean \pm SEM) (group sizes n=8-10) (*p<0.05)

LCGU study	Treatment	Residual plasma 2DG (nCi/ml)	Blood glucose (mg/dl)	Plasma corticosterone (ng/ml)
Multiple-Duration Restraint	Control	44 \pm 3	135 \pm 9	65 \pm 15
	2 min	57 \pm 12	163 \pm 9	127 \pm 23 *
	5 min	57 \pm 10	154 \pm 12	112 \pm 17 *
	10 min	49 \pm 3	136 \pm 11	146 \pm 23 *
	20 min	50 \pm 3	154 \pm 8	188 \pm 18 *
	45 min	49 \pm 6	144 \pm 22	323 \pm 11 *

Discussion

The present studies aimed to compare the effects of an acute stressor, in this case restraint, to the effects of manipulating the CRF system on LCGU and the HPA axis, as described in the previous chapter.

Restraint stress activated the HPA axis, in a duration-dependent manner, indicated by increased levels of plasma corticosterone. This activation of the HPA axis after restraint stress agrees with reports from the literature (Crine et al., 1983; Odio and Maickel, 1985; Hauger et al., 1988; Rowland and Dunn, 1995; Torres et al., 2001; Rivalland et al., 2007), is similar to that seen after central administration of CRF or the related endogenous peptides Urocortin 1, 2 and 3, and indicates the activation of the CRF system at least at the level of the paraventricular nucleus of the hypothalamus (PVN).

However, in contrast to the apparent CRF₂-mediated increases in LCGU demonstrated after the administration of CRF and Urocortin 1, LCGU was reduced after 45 minutes of restraint stress by up to 42 % (in the cerebellum). This is in agreement with previous reports in the literature (Soncrant et al., 1988; Xing et al., 1990), but indicates that restraint stress-induced changes in LCGU are unlikely to be mediated by a similar mechanism involving CRF₂. The failure of the CRF₁ antagonist R278995 to ameliorate the effects of restraint stress on LCGU suggests that in this case this receptor is not the primary component in the LCGU response, perhaps implicating other neurotransmitter systems, discussed further below.

Many areas involved in stress processing receive noradrenergic innervation, generally arising from hindbrain regions and projecting to limbic and cortical regions (Cunningham, Jr. and Sawchenko, 1988). As a result of this widespread innervation many stressful stimuli will stimulate noradrenergic activity in stress-related areas (Pacak et al., 1995b). For example, noradrenaline release occurs in the hippocampus in response to restraint stress (Vahabzadeh and Fillenz, 1994) and several other stressors will increase noradrenaline release in the medial prefrontal cortex (mPFC), including immobilisation, novel environment and conditioned fear (McQuade and Stanford, 2000; Swanson et al., 2004).

Furthermore, any one particular stressor will cause noradrenaline release across several stress-related areas. For example, microdialysis studies have shown that electric footshock induces noradrenaline release in the PVN (Yokoo et al., 1990; Ishizuka et al., 2000), amygdala (Quirarte et al., 1998; Williams et al., 1998), hippocampus (Hajos-Korcsok et al., 2003) and mPFC (Ishizuka et al., 2000), encompassing a number of the regions exhibiting reduced LCGU in the present studies.

Activation of the serotonergic system also occurs during stressor exposure, and a variety of stressors can evoke release or turnover of 5HT in the PFC, hippocampus, amygdala, nucleus accumbens and LC (Pei et al., 1990; Shimizu et al., 1992; Kawahara et al., 1993; Inoue et al., 1994; Linthorst et al., 1995; Adell et al., 1997; Amat et al., 1998; Kaehler et al., 2000; Funada and Hara, 2001), which may influence LCGU. Indeed, acute inhibition of central serotonin reuptake using the selective serotonin reuptake inhibitor (SSRI) fluoxetine has been reported to decrease LCGU in a widespread manner throughout the rat brain (Freo et al., 2000). Evidence for interactions between the serotonergic and CRF systems, particularly in the dorsal raphe (DR) (Price et al., 1998, and briefly reviewed in Chapter 1) also supports the possibility that while the present studies do not directly implicate CRF receptors in restraint stress-induced changes in LCGU, they may still be involved to some degree.

The acute stress of restraint or footshock has been reported to activate the mesolimbic dopamine system (Puglisi-Allegra et al., 1991), and restraint stress has also been reported to increase concentrations of the dopamine metabolite dihydroxyphenylacetic acid (DOPAC) in the PFC and accumbens, and induce Fos immunoreactivity in dopamine neurons of the VTA (Deutch et al., 1991). However, mixed effects on LCGU, with increases in some regions, such as the substantia nigra, have been reported after direct stimulation of dopamine receptors (Levant et al., 1998), which may suggest that this system could also be limited to a minor role in restraint stress-induced LCGU changes.

Both restraint stress and swim stress have been reported to induce widespread release of glutamate in the mPFC, hippocampus, striatum and nucleus accumbens (Moghaddam, 1993). However, the mechanistic links

between glutamate and LCGU suggest that this may not be directly linked to the effects seen on LCGU in the present studies, as glutamate stimulates the glycolytic processing of glucose in astrocytes, which is indicated by increased LCGU (Pellerin and Magistretti, 1994), rather than the reductions seen presently after restraint stress.

The near ubiquitous inhibitory effect of GABA on neural activity has made it difficult to attribute the effects of this neurotransmitter system on specific aspects of the stress response (Ingram, 2005). However increased GABA has been measured in the LC after immobilisation stress (Singewald et al., 2000) and in the amygdala in response to noise (Singewald et al., 1995), and furthermore strong reductions in LCGU have been reported after chronic infusion of GABA into the nucleus basalis magnocellularis (Majchrzak et al., 1992), indicating one possible mechanism for the reductions in LCGU seen in the present studies.

While in the first of the present studies restraint stress significantly reduced LCGU in all dissected regions, significance was only reached in the second study in four of the seven regions. This discrepancy may be explained by the differing experimental design between the two studies, necessitating different methods of statistical analysis.

LCGU was reduced in the dissected frontal cortical region. Evidence suggests that the mPFC plays an important role in regulating the HPA response to emotional stress (Diorio et al., 1993; Figueiredo et al., 2003; Spencer et al., 2005). From lesion studies, the literature generally suggests that the mPFC influences on stress-induced HPA responses are inhibitory (Diorio et al., 1993; Brake et al., 2000; Figueiredo et al., 2003; Spencer et al., 2005), although some studies suggest altered basal but not stress-induced HPA responses after lesioning of the mPFC (Sullivan and Gratton, 2002a), which also suggests a role for this region in basal HPA axis regulation. Furthermore, the role of the mPFC in modulating HPA activity appears to be subregion specific, as lesions of the dorsal mPFC led to exaggerated HPA responses to stress, but ventral mPFC lesions led to mildly suppressed HPA responses (Radley et al., 2006).

In studies of LCGU in response to other stressors, effects on LCGU other than reductions have been reported. In agreement with the present, and reported, data for restraint stress, reduced LCGU has been reported during hypothermia (Frietsch et al., 2000) in all regions measured. Although hypothermia was induced under anaesthesia, which itself affected LCGU, the changes found were still significant when compared to an anaesthetised normothermic control group. However, increases in LCGU after swim stress have been reported in prefrontal cortical areas, motor cortex and lateral septum, and these effects matched with increased fos-like-immunoreactivity in these areas (Duncan et al., 1993). Differences in the physical context of the stressor could explain such contradictory findings. For example, restraint/immobility and hypothermia (in the context above) could be considered to reflect stressors with (imposed) limited physical activity, while clearly forced swim stress requires a large activity component. Interestingly, the forced swim stress-induced increase in LCGU in the lateral septum was blocked by treatment with imipramine (Duncan et al., 1993), which in this acute context implicates the serotonergic and noradrenergic systems in these LCGU changes.

Contrasting effects on LCGU have also been reported after treatment with pharmacological stressors, perhaps in a species-dependent manner. Injection of the pharmacological stressor FG7142, a benzodiazepine partial inverse agonist, in rhesus monkeys reduced LCGU in all regions measured (including the frontal cortex, thalamus and cerebellum), and plasma cortisol levels were increased (Takamatsu et al., 2003a) and reduced LCGU after FG7142 has been reported in anxious humans (Gur et al., 1987). Also in rhesus monkeys, the anxiety-inducing agent mCPP induced global reductions in LCGU (Takamatsu et al., 2003b). In contrast, FG7142 administration in rats increased LCGU in predominantly limbic structures (Ableitner and Herz, 1987), and the anxiolytic diazepam decreased LCGU in matching regions (Ableitner et al., 1985). FG7142 has also been reported to increase c-fos mRNA expression in rats in regions including frontal cortical regions, accumbens, medial septum and the ventral hippocampus (Funk et al., 2006), matching those regions with altered LCGU in reported and present studies, although c-fos techniques can only indicate a response in immediate-early gene transcription, and thus cannot help

differentiate the contrasting increases or decreases in LCGU seen after FG7142 administration.

LCGU can also be estimated by measurement of extracellular lactate release (Kuhr and Korf, 1988), using *in vivo* microdialysis. As discussed in the General Introduction, lactate release after glycolytic processing of glucose in astrocytes is a likely cellular mechanism quantified in the measurement of LCGU (Pellerin and Magistretti, 1994). It has been shown that lactate release in the rat medial prefrontal cortex is immediately and transiently increased by immobilisation for 5 min, and indeed also after noise or tail-pinch stressors (Takita et al., 1992). During 90 min of restraint lactate release was increased initially (approx 15 min) before falling gradually to baseline levels. Increased LCGU (as estimated by lactate release) during the first minutes of immobilisation stress may reflect an active coping response, while a reduction in LCGU after continuous restraint (as measured in the present studies using 2DG) may reflect an inability of an active coping response to manage continued restraint, perhaps even reflecting a change to passive coping. The effect of stress on the HPA axis would not appear to be affected by such a differential mechanism, as attested to by the clear restraint-duration-dependent gradual increase in plasma corticosterone.

Glucocorticoid feedback may play a role in LCGU changes during activation of the HPA axis, particularly in regions associated with the HPA axis negative feedback loop, such as the hippocampus (Herman et al., 1992; Cullinan et al., 1993). A classic catabolic action of glucocorticoids in numerous peripheral tissues is to inhibit glucose uptake into cells (Munck, 1971; Horner et al., 1990), and studies have shown that in hippocampal cell cultures glucocorticoids significantly inhibit glucose uptake and oxidation both by neurons and astrocytes (Horner et al., 1990; Virgin, Jr. et al., 1991), although exposure for at least 4h appears to be necessary. LCGU was increased following acute adrenalectomy in rats, further indicating a negative modulatory role of glucocorticoids on cerebral glucose metabolism (Kadekaro et al., 1988), and acute hydrocortisone challenge has been shown to reduce hippocampal LCGU in elderly patients (de Leon et al., 1997). Whether such a mechanism is involved in the LCGU changes after restraint stress in the present studies is unclear. While reduced hippocampal

LCGU with simultaneous increased plasma corticosterone is in agreement with the hypothesised mechanism, the increased hippocampal LCGU with concomitant increased plasma corticosterone after CRF treatment as described in the previous chapter is in direct contrast with this theory. Indeed this may highlight a drawback of the LCGU technique. It is possible that glucocorticoid feedback does induce a reduction of LCGU in a specific subset of hippocampal neurons, however when LCGU is measured in the complete hippocampus, increased LCGU in a separate neuronal population after CRF treatment may mask this effect.

Lastly, a proteomic analysis of rat hippocampus after repeated psychosocial stress revealed (among others) changes in proteins involved in energy metabolism, including pyruvate kinase (Carboni et al., 2006), an enzyme involved in the glycolysis of glucose to pyruvate, and thus involved in the one of the likely cellular mechanisms behind the measurement of LCGU (Pellerin and Magistretti, 1994), and thus providing a further link between stress and changes in LCGU.

In conclusion, the present studies suggest that restraint stress does not induce similar effects to CRF or Urocortin 1 on LCGU, indicating differing central mechanisms, although one cannot preclude that the specific LCGU response to CRF₂ receptor stimulation is not a part of a more widespread neurotransmitter system response to stress. The clear HPA axis response to stress, however, indicates the involvement of at least this part of the CRF system in the measured responses to restraint stress.

Chapter 6 – General Discussion

General Discussion

The aim of this thesis was to test the hypothesis that CRF and the related endogenous peptides, Urocortin 1, 2, and 3, induce different patterns of neuronal activation after central administration. This was achieved using modified versions of Sokoloff et al.'s technique for the measurement of local cerebral glucose utilisation (LCGU), and the involvement of CRF₁ and CRF₂ receptors in the LCGU response to CRF was examined using selective antagonists and mutant mice lacking a functional CRF₁ receptor.

The measurement of LCGU provides a brain region specific estimate of neuronal activity (Sokoloff et al., 1977), and the technique can be applied in a translational manner across a range of species, including rodents, primates and humans, through the use of different radioligands.

Two modified versions of the original LCGU technique were chosen after experimental examination of a range of modifications, as described in detail in Chapter 2.

The first of these versions, and the one primarily used for the study of the CRF system, was a protocol using the cheaper, safer, ³H-2-deoxyglucose (2DG), injected intraperitoneally in mice, and with the dissection of a number of brain regions. Reports in the literature suggested that these modifications could reliably be used to measure LCGU, with results strongly correlating to those obtained using the originally described technique (Meibach et al., 1980; Kelly and McCulloch, 1983b), and this was supported by the results of the studies described in Chapter 2. LCGU measured using ³H-2DG correlated highly with that measured by Sokoloff et al. using ¹⁴C-2DG, and furthermore the combination of using the tritiated ligand in mice serves to significantly reduce cost compared to ¹⁴C-2DG in rats. The dissection of specific brain regions does not have the same resolution for small regions as autoradiography, and groups heterogeneous populations of neurons into given dissected regions, but allows the rapid measurement of LCGU without the need for sectioning or the repeated delineation of brain regions after autoradiography.

The second version, used for the verification of important experimental results from the first protocol, utilised the ^{14}C -labelled version of 2DG, also injected intraperitoneally, in order to allow the measurement of LCGU in sections taken from treated mice exposed to film. This protocol allowed the replication of important results via an alternative method of measurement.

When these versions of the LCGU technique were used to study the CRF system, an interesting but complex set of results were obtained. The large (up to 67 % in the cerebellum) CRF-induced increases in LCGU could be abolished by the CRF₂-selective antagonist antisauvagine-30, and LCGU was also increased in a more selective set of brain regions by the CRF-related peptide Urocortin 1, which is postulated to function as the endogenous ligand for the CRF₂ receptor in the hindbrain (Hauger et al., 2003). The involvement of the CRF₂ receptor, but not the CRF₁ receptor to a detectable degree, in the response to CRF was further confirmed in studies using mice lacking a functional CRF₁ receptor and using a selective CRF₁ antagonist. However, neither of the CRF₂-selective CRF-related peptide agonists, Urocortin 2 or Stresscopin, had any effect on LCGU, which may indicate ligand-specific receptor activation, where CRF and Urocortin 1 are capable of stimulating CRF₂ to influence LCGU, but Urocortin 2 and Stresscopin are not, despite their *in vitro* affinity for CRF₂. The concept of ligand-dependent differential regulation of receptor-coupled effector pathways has recently been reviewed (Urban et al., 2007), and specifically includes agonist-specific signaling. It is also possible that an effect of these peptides on LCGU was missed through dosing or time of injection, although a range of doses were tested and delayed behavioural effects have only been reported for Urocortin 2 (Valdez et al., 2002), not Stresscopin. The use of a stressful technique for the ICV injection of CRF and the Urocortins may also have adversely affected the LCGU results obtained, although a clear effect of the ICV injection technique alone on LCGU was not seen. It is possible that the effects of CRF on LCGU seen in the present studies represent a potentiation of stress-induced effects on LCGU brought about by the freehand ICV injection procedure. However, the clear effect of antisauvagine-30 on CRF-induced changes in LCGU continues to implicate CRF₂ receptors in the response, in contrast to the effects of Urocortin 2 and Stresscopin. Furthermore, as discussed and demonstrated in Chapter 5,

many stressors reduce LCGU in numerous brain regions, which could suggest that CRF-potential of stress effects on LCGU could be expected to further reduce LCGU, in contrast to the increases found in the present studies. The extent of such an effect could be investigated through the replication of the key elements of the present data using pre-implanted cannulae for ICV injection, which under well-controlled conditions constitute a minimal stressor for the animal.

Thus, in addition to the numerous endogenous CRF-related peptides, acting on at least two central CRF receptor subtypes, the further complication of the CRF system through postulated ligand-specific effects, may help to explain the somewhat unclear literature surrounding the function of the CRF system in the brain. While the regions examined in these studies are all sites of CRF peptide or receptor expression, these regions are also likely to have an involvement in stress-related functions. Whether the present data represent direct CRF-related peptide-induced changes in LCGU or a potentiation of ICV-injection stress-induced changes in LCGU, changes in neuronal activation in aspects of the dissected hindbrain region, for example the LC, PPTg and aspects of the medulla, suggest links to arousal, attention and autonomic regulation, in all of which CRF has been implicated (Dunn and Berridge, 1990, and reviewed in Warnock et al., 2006). Increased LCGU in the amygdala and hippocampus may relate to their roles in the regulation of the responses to stressful situations, while the LCGU changes in the septum and frontal cortex may be of functional consequence in stress coping and the termination of stress responses.

The effect of the common stressor, restraint, on LCGU was studied for comparison to the effects of CRF on LCGU, as the CRF system has been implicated in the stress response (Funk et al., 2003; Crane et al., 2005; Funk et al., 2006; Rivalland et al., 2007), particularly for its role in the HPA axis. In contrast to the increases in LCGU seen after CRF or Urocortin 1 administration, restraint stress reduced LCGU in all regions dissected, an effect that was unaffected by pre-treatment with a selective CRF₁ antagonist (at a dose corresponding to approximately 75% occupancy in the frontal cortex (Chaki et al., 2004)). This contrasting response suggested that a mechanism similar to the

CRF-induced effects on LCGU involving CRF₂ is unlikely, and the failure of the CRF₁ antagonist to ameliorate the LCGU response to restraint suggested that this receptor may not be a principal component of the response, but is perhaps part of a response involving numerous neurotransmitter systems. However, as the studies into the effects of restraint stress on LCGU did not require the administration of compounds by ICV injection, it may be useful to further investigate the comparability of the present studies between CRF-related peptide-induced effects and restraint stress-induced effects on LCGU.

Despite the opposing effects on LCGU seen after CRF administration and restraint stress, both restraint and the CRF-related peptides had an activating effect on the HPA axis, indicating at least one shared component in the response to these treatments. In contrast to the differing effects of the CRF-related peptides on LCGU, the HPA axis was further activated above the level induced by ICV injection stress by not only CRF and Urocortin 1, but also by Urocortin 2 and Stresscopin (dose-dependently), indicating that activation of the HPA axis can be mediated by either the CRF₁ or CRF₂ receptor. The role of CRF₁ in activating the HPA axis is well established (reviewed in the Introduction), and more recently evidence supporting a role of CRF₂ has also been presented in the literature (de Groote et al., 2005; Maruyama et al., 2007). Based on the distribution of CRF receptors in the hypothalamic paraventricular nucleus (PVN) and the anterior pituitary, the two receptor subtypes may exert their influence at different levels of the HPA axis. While CRF₁ is the predominant subtype expressed in the anterior pituitary, CRF₂ is the more highly expressed of the two subtypes at the level of the PVN (Chalmers et al., 1995). In addition, the activation of the HPA axis by Urocortin 2 and Stresscopin despite no effects on LCGU, the continued effect of CRF administration on the HPA axis despite abolition of this peptide's effects on LCGU by antisauvagine-30, and indeed the restraint stress-induced activation of the HPA axis despite an opposite effect on LCGU to that of CRF, combine to suggest that the HPA axis can be uncoupled from those central effects measured through LCGU.

Further studies investigating the possibility of ligand-specific receptor activation and second messenger pathways within the CRF system may help to draw more detailed conclusions from the present data. Such mechanisms will

most likely be best studied *in vitro* initially, in order to more readily isolate the second messenger systems likely to be involved. The differences in calcium signaling in different cell lines expressing CRF receptors is currently being investigated (Dautzenberg et al., 2004), although the translation of these effects to the endogenous situation is hampered the relative lack of cell lines endogenously expressing the CRF₂ receptor (Gutknecht, personal communication).

Interactions with other neurotransmitter systems, such as the serotonergic and noradrenergic systems, in the LCGU response to the CRF-related peptides could be investigated using antagonists selective for components of these systems. While the LCGU response in some regions may be a direct effect of CRF receptor stimulation, the large body of evidence for interactions between the CRF system and other neurotransmitter systems (as discussed in the Introduction) tends to suggest that complex interactions, some within specific brain regions, could be involved in the effects of CRF on LCGU. The involvement of specific brain regions in the LCGU response could also be investigated using specific lesion studies.

While the modified versions of the LCGU technique used here do not allow the measurement of changes in LCGU during periods less than 45 minutes, it may be possible to study the temporal course of changes in LCGU produced by CRF-related peptides or restraint stress by measuring extracellular lactate release using microdialysis. This technique can be used to estimate LCGU (Kuhr and Korf, 1988), and is capable of sampling extracellular lactate levels with improved temporal resolution, compared to the measurement of LCGU using 2DG.

In conclusion, the studies comprising this thesis have confirmed the hypothesis that central administration of CRF and the Urocortins induces differing patterns of neuronal activation, measured through local cerebral glucose utilisation. The CRF₂ receptor was clearly implicated in the mediation of CRF-induced increases in LCGU, and a number of brain regions exhibited altered LCGU after CRF administration, highlighting the need to further study the role of

these regions in the CRF system's involvement in stress-related disorders such as post-traumatic stress disorder and depression.

Appendices

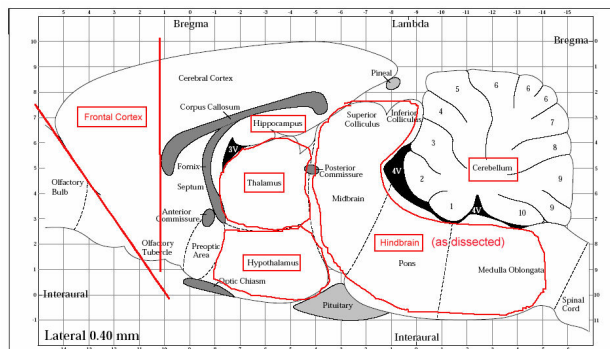
Appendix 1

Brain Region Dissection Illustrations

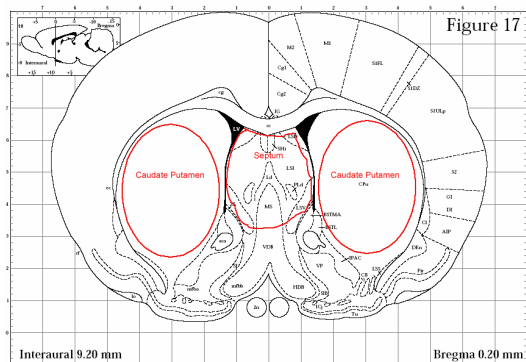
Figure 43: Diagrams illustrating the definition of brain regions for dissection.

a) Sagittal definition for frontal cortex, hippocampus, thalamus, hypothalamus, hindbrain and cerebellum. b) Coronal definition for septum and caudate putamen. c) Coronal definition for thalamus, hypothalamus and temporal region.

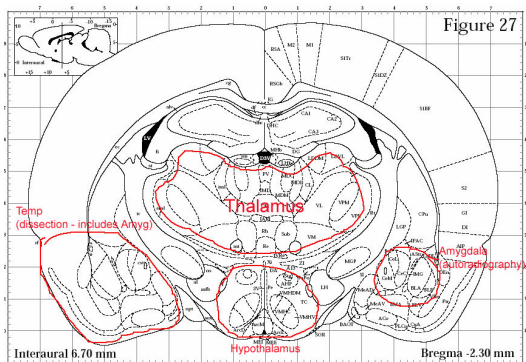
a)



b)



c)



Appendix 2

Analysis of CRF₁ and CRF₂ mRNA expression in CRF1KO mice

This study was carried out to confirm the lack of functional CRF₁ receptor expression in CRF₁ knockout mice that were used to examine the role of CRF₁ in the LCGU response to CRF.

Methods

The following analysis was carried out by Ilse Goris (Johnson & Johnson Pharmaceutical Research & Development). Tissue from different brain regions was disrupted and homogenized using a Mixer Mill MM300 (Quiagen). RNA was isolated using an RNeasy Lipid Tissue kit (Qiagen) and First strand cDNA synthesis was performed with SuperScript II Reverse Transcriptase (Invitrogen) followed by Real time RTQ. Real Time Quantitative RTQ-PCR was performed using an ABI PRISM 7900 Sequence Detection System instrument (Applied Biosystems) using Assay on Demand primers (Applied Biosystems). In order to identify whether a truncated transcript of CRF₁ was still expressed in the mutants, two primer-probe sets for CRF₁ were used, one targeting the deleted exons 8-9 (Mm00432675_m1, Applied Biosystems) and another targeting the non-deleted exons 3-4 (Mm00432670_m1, Applied Biosystems). For CRF₂ a primer-probe set was used targeting exon 13:

Forward primer: GGGAGAACAGAAGCGCCTG

Reverse primer: CCCTTGTTTCAATCACTCCCA

Probe: AGAAGGGTGAGGATCCCCCAAATCAGAGT[5']6_FAM [3']TAMRA

mRNA expression was expressed as a ratio of CRF₁ or CRF₂ expression against beta-actin expression.

Results

Using the probe set for the deleted exons, 8-9, of CRF₁, no expression of CRF₁ mRNA was found as compared to wild-type littermates (Figure 44). In

contrast, the probe set for exons 3-4 revealed expression of a CRF₁ mRNA-like signal similar to that in wild-types (Figure 45).

CRF₂ mRNA expression was unaltered between CRF1KO and wild-type littermates (Figure 46).

Figure 44: Expression of CRF₁ receptor mRNA in CRF1KO (KO) and wild-type (WT) mice as measured using a probe set for exons 8-9.

(N.B. the CRF₁:beta-actin ratio in KO mice was precisely zero, thus is not apparent as a column in the Figure below).

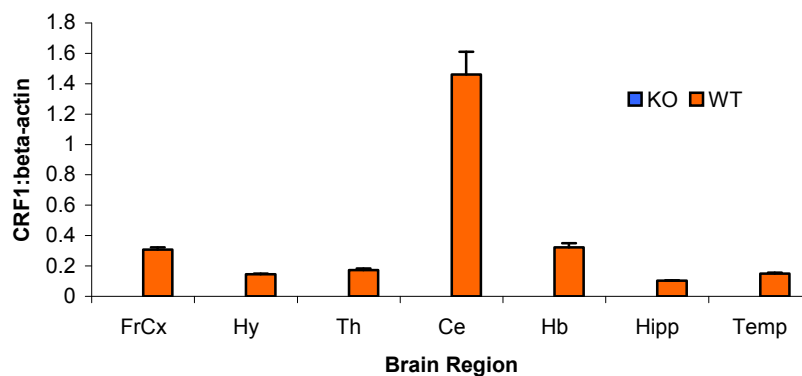


Figure 45: Expression of CRF₁ receptor mRNA in CRF1KO (KO) and wild-type (WT) mice as measured using a probe set for exons 3-4.

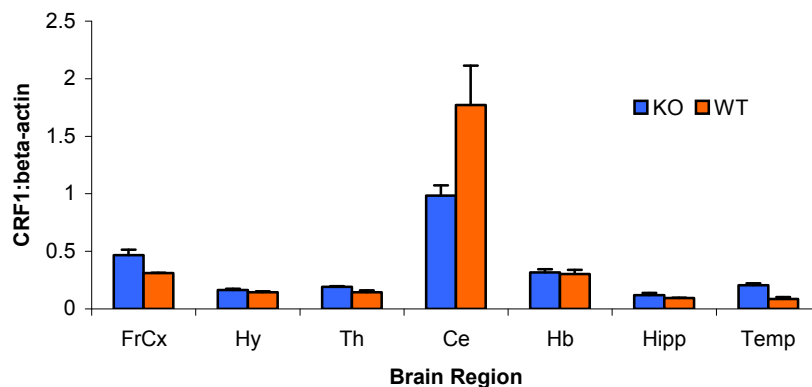
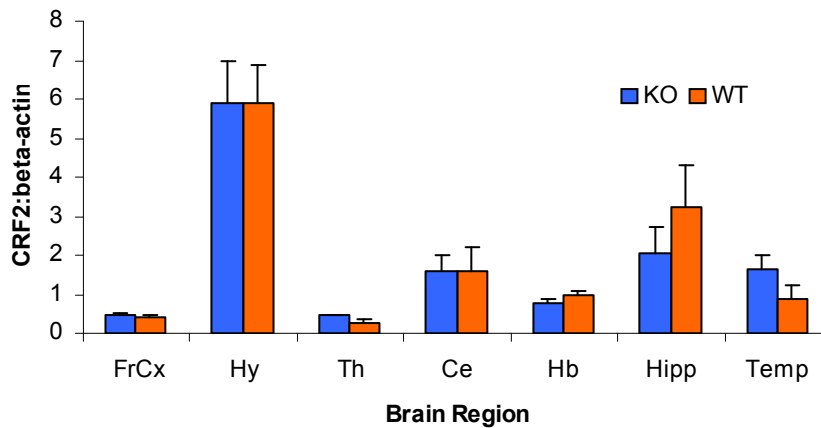


Figure 46: Expression of CRF₂ receptor mRNA in CRF1KO (KO) and wild-type (WT) mice as measured using a probe set for exon 13.



Discussion

Analysis of CRF₁ mRNA expression in CRF1KO mice confirms the knock out of exons 8-9 of the CRF₁ gene. However, the second probe set targeting exons 3-4, which were not targeted in the generation of these CRF₁ knockout animals, revealed that a truncated transcript of the receptor mRNA is still expressed. As exons 8-9 encode a part of the receptor binding domain (Timpl et al., 1998), this truncated transcript can be considered inactive. Furthermore, the large reduction in HPA axis activation after the ICV injection of CRF in CRF1KO mice (Chapter 4, Table 15) supports the expected phenotype (Timpl et al., 1998).

The unaltered expression of CRF₂ mRNA in CRF1KO mice may indicate that any compensatory processes do not affect the expression of this CRF receptor subtype.

In conclusion, the measured expression of CRF₁ and CRF₂ receptor mRNA confirms the CRF₁ knock-out status of these mice.

Appendix 3

Administration of R278995 in the diet

This pilot study was carried out to confirm the suitability of dietary administration to deliver the CRF₁ antagonist R278995 (Chaki et al., 2004) in sufficient quantity to occupy CRF₁ receptors in the mouse brain.

Methods

Basic protocol

In initial LCGU studies it became clear that a preinjection (vehicle or compound) disrupted the LCGU response to CRF in the positive control group, making it impossible to study the effect of the CRF₁-selective antagonist R278995 on this response via this method. For this reason, it was decided to administer the compound through the diet.

The dry compound was administered to male C57Bl/6 mice in a powdered premixed diet from Research Diets (<http://www.researchdiets.com>) to an approximate dose of 40 mg/kg/day based on mice consuming approximately 4 grams per 24 hours (as indicated by pilot studies). The final diet was D12450B.

The standard VRF1 diet was replaced with the powdered diet containing R278995 for 5 days prior to testing. Plasma and brain levels of R278995 were measured by Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC-MS/MS) on day 6 at three time points (0800, 1100, 1500) to assess the fall in levels based upon the 1.8 hour plasma half-life of the compound, and taking into consideration that food consumption effectively stops during the light-phase (confirmed in a separate pilot study). The occupancy of CRF₁ receptors in the frontal cortex was also measured on day 6 using an ex vivo receptor binding protocol utilizing ¹²⁵I-sauvagine. Both plasma/brain compound levels and CRF₁ occupancy were compared to those after acute oral administration of 40 mg/kg R278995 (1 hour preinjection).

LC-MS/MS for measurement of compound in plasma and brain

LC-MS/MS was carried out by the staff of the ADME-Tox department of Johnson & Johnson Pharmaceutical Research & Development. Brain samples (whole brain) were homogenized in mQ-water (1:10 ratio by weight). After solubilisation (with methanol) and protein precipitation (with acetonitrile), plasma and brain samples were quantified on an reversed phase liquid chromatography (LC)-column (BDS hypersil C18 3 μ m, 50x4.6 mm; Thermo). Mobile phases consisted of acetonitrile (solvent A) and 0.01M ammonium formate (pH4) (solvent B). Chromatographic separation was obtained by gradient elution (10 % solvent A; 90 % solvent B starting conditions to 90 % solvent A; 10 % solvent B in 3 min) at a flow rate of 1.2 ml/min.

LC-MS/MS analysis was carried out on a API-3000 MS/MS (Applied Biosystems, Toronto, Canada), which was coupled to an HPLC-system (Agilent; Palo Alto, US). The MS/MS, operated in the positive ion mode using the TurbolonSpray™-interface (electrospray ionisation), was optimised for the quantification of the compound (MRM transition for R278995: 412.1 > 315).

The limit of quantification was 5.00 ng/ml for plasma samples and 50.0 ng/g for brain samples. The accuracy (intra batch accuracy from independent QC samples) was between 80% and 120% of the nominal value over the entire range for plasma and brain samples.

Measurement of CRF₁ occupancy in frontal cortex

Occupancy of CRF₁ receptors was measured in 20 μ m frontal cortex sections taken on a Leica cryostat. The slides were pre-incubated in rinsing buffer (50 mM Tris-HCl, 2 mM EGTA, 10 mM MgCl₂, pH 7.4) for 1 minute before incubation for either total or non-specific binding. Incubation buffer was prepared by the addition of 4 μ g/ml leupeptin, 2 μ g/ml chymostatin, 40 μ g/ml bacitracin, 1 mg/ml bovine serum albumin, and finally ¹²⁵I-sauvagine (final concentration 60258 cpm/50 μ l) to the rinsing buffer. Non-specific binding was assessed by the

addition of the CRF₁ antagonist CRA5626 (10^{-5} M) (Steckler et al., 2006) to an aliquot of the incubation buffer. After 45 min incubation for either total or non-specific binding, the slides were washed in rinsing buffer for 5 min in triplicate and finally rinsed in distilled water before air drying. The sections were exposed to Ektascan films (Kodak) for 10 days with ¹²⁵I-standards, and the resultant autoradiograms were analysed and quantified using a MicroComputer Imaging Device (MCID) M1 imaging system. Ex vivo receptor labelling in R278995 treated animals was expressed as a percentage of sauvagine binding in control animals.

Results

R278995 was clearly detectable in both plasma and brain after 5 day dietary administration (Figure 47), although at levels up to ten fold lower than those seen after acute oral administration (Figure 48). The level of R278995 in the plasma after dietary administration declined with time throughout the test day (light phase). In contrast, a similar decline in brain R278995 levels was not seen.

Despite the large difference in detectable plasma and brain compound levels between dietary and acute oral routes, CRF₁ receptor occupancy was similar in acute oral and 0800 sampled dietary groups (Figure 49). Occupancy declined by the later (1100) sampling time.

Figure 47: Plasma and brain levels of R278995 after 5 days dietary administration at 40 mg/kg/day

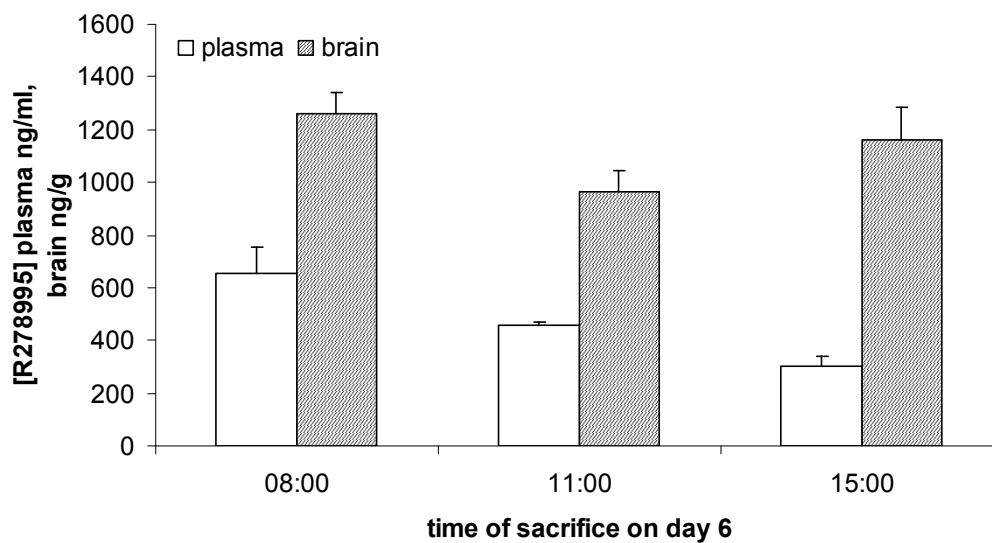


Figure 48: Plasma and brain levels of R278995 1 hour after oral administration of R278995 at 40 mg/kg

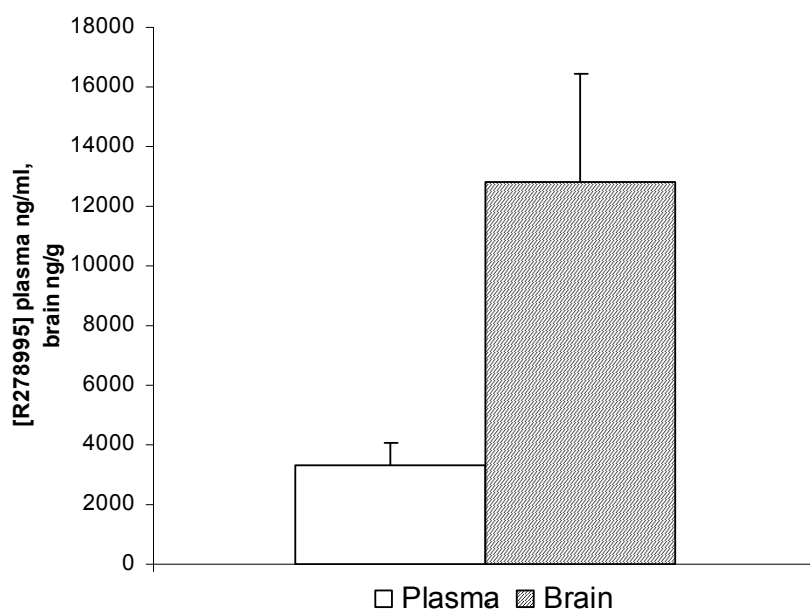
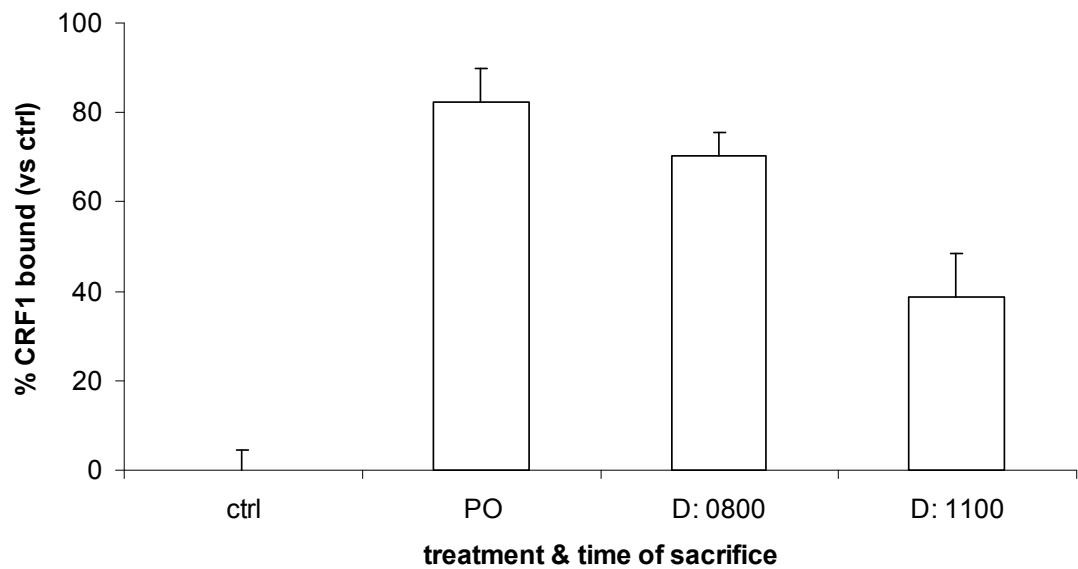


Figure 49: Ex vivo CRF₁ receptor binding after either acute oral or 5 day dietary administration of R278995 (Key: ctrl – untreated control group; PO – oral dosing; D – dietary dosing)



Discussion

This study demonstrated that administration of R278995 in the diet produced detectable levels of the compound in plasma and brain, with an occupancy of CRF₁ receptors similar to that after acute administration of the same compound.

Plasma levels of R278995 declined with testing time. The testing times selected all fall within the light-phase, the period in which later studies would be carried out. An initial pilot study confirmed that C57Bl/6 mice consume approximately 4 g of powdered diet during the dark-phase, but only approximately 0.5 g during the light-phase. Thus, based upon the 1.8 hour plasma half-life of R278995 (internal communication), a fall in plasma compound level during the light-phase was expected.

In contrast, brain compound levels did not exhibit a similar decay. This is supported by initial internal metabolic studies of R278995, which indicated a high blood-brain barrier permeability and increased half-life in this organ.

While the plasma and brain levels of R278995 after dietary administration were substantially lower than those after acute oral administration, the occupancy data indicates that a similar level of receptor binding compared to control animals was obtained. Despite the fall in CRF₁ occupancy at the later morning time point, it was decided that the receptor occupancy during the testing period used in subsequent studies (0730 to 1130) was sufficient to assume antagonism at CRF₁.

In conclusion, dietary administration of R278995 was deemed to be a suitable route for the use of this compound to study the role of CRF₁ in the LCGU response to CRF, in order to avoid the disruptive effect of an acute pre-injection on the LCGU response.

Appendix 4

Publications

Content from this thesis was presented in Abstract and Poster form at the following conference:

- 2007 British Association for Psychopharmacology Summer Meeting, Oxford, UK

23rd-26th July 2006

CRF-induced increases of relative local cerebral glucose utilisation in the mouse brain are mediated by the CRF₂ receptor

Geoff Warnock, Dieder Moechars & Thomas Steckler

Corticotropin-releasing factor (CRF) is well known for its role in the hypothalamic-pituitary-adrenocortical (HPA) axis and its involvement in stress and anxiety, among other centrally mediated functions. CRF acts via two main receptor subtypes, CRF₁ and CRF₂. Other endogenous CRF-related peptide ligands for the two primary CRF receptors are the urocortins 1, 2 and 3. While CRF is thought to mediate its anxiolytic-like properties through CRF₁, the role of CRF₂ and its endogenous ligands urocortin 2 and 3 are unclear in the literature.

Measurement of local cerebral glucose utilisation (LCGU) provides an estimate of neuronal activity in specific regions of the brain. We have previously shown that CRF (and urocortin 1) increase LCGU after intracerebroventricular (ICV) administration, while simultaneously activating the HPA axis. Both of these peptides are non-specific for either CRF₁ or CRF₂, although CRF has an approximately 28-fold greater affinity for CRF₁.

The present studies examine the role of CRF₁ and CRF₂ in the CRF-induced LCGU response, using a modified version of the LCGU technique, in which ³H-2-deoxyglucose (2DG) was injected intraperitoneally in adult male C57BL/6N mice or CRF₁-null mutant mice (CRF1KO) and wild-type littermate controls. Selected brain regions were dissected 45 minutes after simultaneous administration of

peptides and 2DG. Following homogenisation, 2DG levels were measured by liquid scintillation counting.

The involvement of CRF₁ was examined by measuring CRF-induced changes in LCGU in CRF1KO and wild-type mice, while the involvement of CRF₂ was examined by co-administering the CRF₂-selective antagonist antisauvagine-30 (aSVG) in C57BL/6N mice.

0.5µg CRF increased LCGU in thalamic, cerebellar, hindbrain and hippocampal regions in both CRF1KO mice and their wild-type littermates compared to saline-treated controls (n=7-8/group), while the CRF-induced increase in plasma corticosterone in wild-types was greatly diminished in CRF1KO mice. In C57BL/6N mice (n=6-8/group), 1µg CRF significantly increased LCGU in frontal cortical, thalamic, cerebellar, hindbrain and hippocampal regions. This effect was completely abolished by aSVG (1 and 3µg) co-administered with CRF.

The fact that CRF was effective in mice lacking a functional CRF₁ receptor suggests that the LCGU response is not CRF₁ mediated. The abolishment of CRF-induced increases in LCGU by the CRF₂-selective antagonist aSVG clearly suggests a role for this receptor in the response. Continuing studies using a selective CRF₁ antagonist and co-administration of aSVG with CRF in CRF1KO mice will further characterise the complicated interaction between CRF₁ and CRF₂ in cerebral glucose utilisation.

Also published during the course of this thesis:

Interactions between CRF and acetylcholine in the modulation of cognitive behaviour

Geoff Warnock, Jos Prickaerts & Thomas Steckler

In: Neurotransmitter Interactions and Cognitive Function (pp. 41-63), Edited by Edward D. Levin, © Birkhäuser Verlag/Switzerland

PMID: 17019882 [PubMed - indexed for MEDLINE]

CRF-induced Increases Of Relative Local Cerebral Glucose Utilisation In The Mouse Brain Are Mediated By The CRF2 Receptor

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Introduction

- Corticotroph-releasing factor (CRF) is well known for its role in the hypothalamic-pituitary-adrenocortical (HPA) axis and its involvement in stress and anxiety, among other centrally mediated functions
- CRF1 is clearly implicated in the HPA axis response to stress, and in the mediation of stress- and anxiety-related behaviour, while the role of CRF2 has not yet been clarified. Studies suggest that the role of CRF2 may vary between brain regions and even between specific neurons within a given region
- CRF is an agonist at both CRF1 and CRF2 receptors, although it has approximately 25-fold greater affinity for CRF1
- Measurement of local cerebral glucose utilisation (LCGU) using radiolabelled 2-deoxyglucose (2DG) provides an estimate of neuronal activity in specific regions of the brain (Sokoloff et al), and is thus a useful tool to compare the response to manipulation of the CRF system in the brain
- We have previously shown that ICV administration of CRF increases LCGU

Aim

- To examine the role of the CRF1 and CRF2 receptors in the LCGU response to ICV CRF

Methods

- CRF1 knockout mice were developed in collaboration with Lexicon Genetics, Inc. in a similar fashion to those generated by Triml et al (1998). Cre-LoxP manipulation was used to remove the last 5 exons of the CRF1 gene, resulting in expression of a non-functional protein
- Male mutant and wild-type littermates or C57BL/6 mice were injected with CRF(0.5 or 1µg), or a mixture of CRF (1µg) and the CRF2-specific antagonist antisauvagine-30 (aSVG, 1 or 3µg), using a freehand i/cv injection technique adapted from Peleymounter et al. (2000)
- Immediately following i/cv injection, mice were injected i/p with 300 µCi/kg 2DG, and returned to their homecage for 45 min, until decapitation
- Trunk blood was collected for measurement of corticosterone and ACTH, and the brains rapidly dissected into regions of interest for homogenisation, and metabolised 2DG content measured by scintillation counting for calculation of relative LCGU
- Treatment effects in individual brain regions were analysed using 2-way ANOVA for the KO study or the Kruskal-Wallis test followed by the Mann-Whitney-U test for post-hoc comparisons (* indicates p<0.05, ** p<0.01, *** p<0.001) for the aSVG study

Conclusion

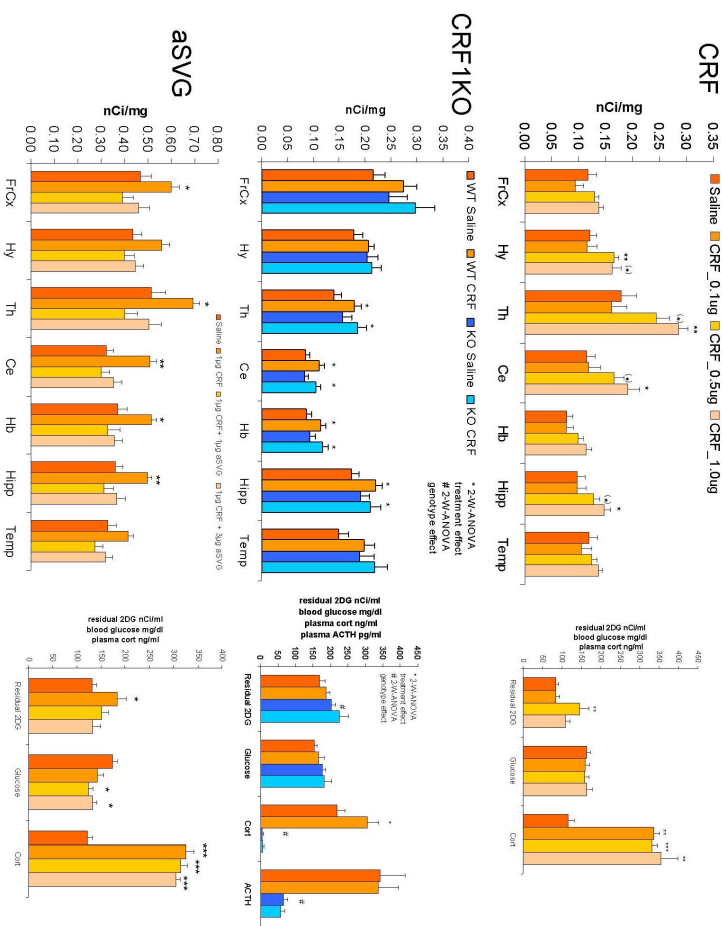
- Failure of CRF1 KO to affect LCGU response to CRF suggests that the response is not CRF1-mediated
- Complete abolishment of the response by aSVG clearly implicates CRF2
- Small effect of aSVG on blood glucose supports a role of CRF2 in energy balance
- Separation of HPA and central effects is suggested by aSVG failure to inhibit CRF-induced corticosterone response, despite abolishing LCGU response

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Results

- CRF induced increases in LCGU in both wild-type and KO mice, but the plasma ACTH and corticosterone response to CRF was greatly reduced in KOs
- aSVG completely abolished the CRF-induced increases in LCGU, but did not affect the plasma corticosterone response



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